Bioavailability of orange juice (poly)phenols: the impact of short-term cessation of training by male endurance athletes

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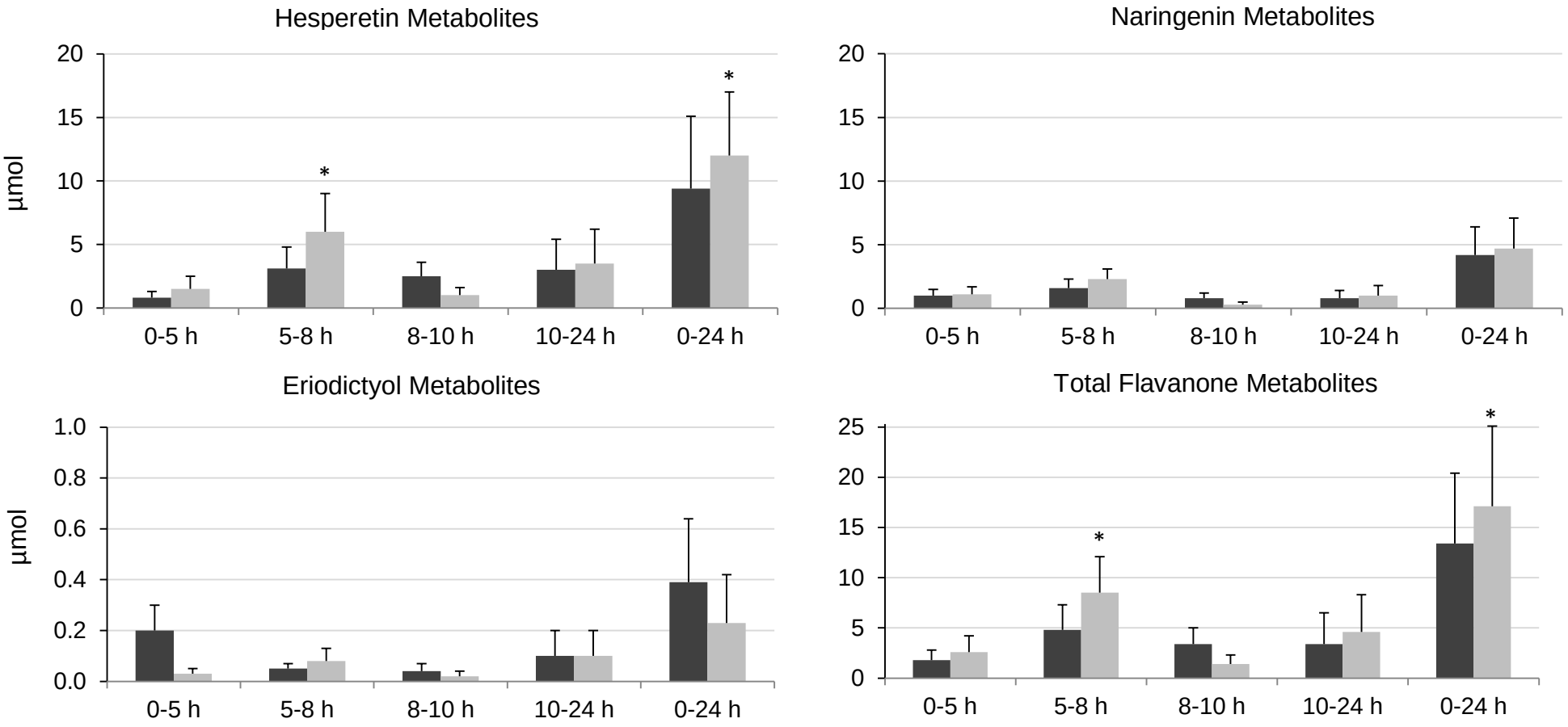
Running heading: Exercise and bioavailability of flavanones

Abbreviations: BMI, body mass index; HPLC-PDA-HR-MS, high performance liquid chromatography–photodiode array–high resolution-mass spectrometry; $\dot{V}O_2max$, maximal oxygen consumption.


Hesperetin Metabolites
µmol
20
15
10
5
0
*
*
0-5 h
5-8 h
8-10 h
10-24 h
0-24 h
Naringenin Metabolites
20
15
10
5
0
0-5 h
5-8 h
8-10 h
10-24 h
0-24 h
Eriodictyol Metabolites
µmol
1.0
0.8
0.6
0.4
0.2
0.0
0-5 h
5-8 h
8-10 h
10-24 h
0-24 h
Total Flavanone Metabolites
25
20
15
10
5
0
*
*
0-5 h
5-8 h
8-10 h
10-24 h
0-24 h

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ABSTRACT

Background: Physical exercise has been reported to increase the bioavailability of citrus flavanones.

Objective: To investigate the bioavailability of orange juice (OJ) (poly)phenols in endurance-trained men before and after cessation of training for 7 days.

Design: Ten fit endurance-trained males, with a maximal oxygen consumption of 58.2 ± 5.3 mL/kg/min, followed a low (poly)phenol diet for 2 d before drinking 500 mL of OJ, containing 398 μmol of (poly)phenols of which 330 μmol were flavanones. After the volunteers stopped training for 7 days the feeding study was repeated. Urine samples were collected 12 h pre- and 24 h post-OJ orange consumption. Bioavailability was assessed by the quantitative analysis of urinary flavanone metabolites and (poly)phenol catabolites using HPLC-HR-MS.

Results: While training, 0-24 h urinary excretion of flavanone metabolites, mainly hesperetin-3′-*O*-glucuronide, hesperetin-3′-sulfate, naringenin-4′-*O*-glucuronide, naringenin-7-*O*-glucuronide, was equivalent to 4.2% of OJ flavanone intake. This increased significantly to 5.2% when OJ was consumed after the volunteers stopped training for 7 days. Overall, this trend, although not significant, was also observed with OJ-derived colonic catabolites which after supplementation in the trained state were excreted in amounts equivalent to 51% of intake compared to 59% after cessation of training. However, urinary excretion of three colonic catabolites of bacterial origin, most notably, 3-(3′-hydroxy-4′-methoxyphenyl)hydracrylic acid, did increase significantly when OJ was consumed post- compared to pre-cessation of training. Data were also obtained on inter-individual variations in flavanone bioavailability.

Conclusion: A 7-day cessation of endurance training enhanced, rather than reduced, the bioavailability of OJ flavanones. The biological significance of these differences and, whether or not they extend to the bioavailability of other dietary (poly)phenols, remains to be determined. Hesperetin-3′-*O*-glucuronide and the colonic microbiota-derived catabolite 3-(3´-hydroxy-4´-methoxyphenyl)hydracrylic acid are key biomarkers of the consumption of hesperetin-*O*-glycoside-containing OJ and other citrus products.

Key words: orange juice flavanones, endurance-trained athletes, cessation of training, urinary metabolites and catabolites, biomarkers of hesperetin intake

INTRODUCTION

Citrus fruits and their juices are the principal dietary source of flavanones (1). Population-based data have linked increased citrus fruit consumption to a reduced risk of stroke (2) and some types of cancer (3, 4). There is evidence that this is due to a high flavanones intake (5, 6). Orange juice (OJ) is one of the main dietary sources of flavanones with the major components being hesperetin-7-*O*-rutinoside (hesperidin) and naringenin-7-*O*-rutinoside (narirutin) (1). Other studies have found that regular consumption of OJ brings about an improvement in vascular function (7, 8), inhibits oxidative stress and inflammatory responses (9) and has a positive effect on metabolic, oxidative and inflammatory biomarkers of health status in normal and overweight subjects (10). Furthermore, it has been reported that daily consumption of OJ containing at least 300 mg (~500 μmol) of flavanones for a period of 12 weeks, enhanced the antioxidant defence system, protected against DNA damage and lipid peroxidation, and reduced blood pressure in overweight and obese adults (11). In addition, administration of a flavanone-rich aronia-citrus juice to triathlon athletes for a period of almost 21 weeks decreased isoprostane markers of oxidative stress (12).

An understanding of the metabolic fate of flavanones in the body is a prerequisite for elucidating the mode of action underlying the protective effects of OJ and citrus consumption in general. Ingested flavanones begin to be absorbed as phase II metabolites in the small intestine but ~70% of ingested flavanones reach the large intestine (13) where, as well as continuing to be absorbed as phase II metabolites, they are subjected to ring fission by the action of the resident microbiota and broken down to phenolic catabolites (14-16) which enter the circulatory system, with a portion undergoing phase II metabolism in coloncytes and/or hepatocytes prior to renal excretion (17). In a recent OJ feeding study, in addition to a ~16% urinary recovery of hesperetin and naringenin metabolites, the quantity of colon-derived phenolic compounds

detected in urine was equivalent to ~88% of flavanone intake, demonstrating that flavanone bioavailability is much higher than previously perceived (18).

In the context of physical training status and flavanone bioavailability, it has been reported that urinary excretion of flavanone metabolites by triathletes, after drinking an aronia (5%)-citrus (95%) juice, was ~ 5-fold higher than that of more sedentary volunteers (19). Exercise training, induces several physiological changes including reduced whole bowel transit time (20) and enhanced muscle blood flow (21), which would be expected to reduce rather than increase flavanone bioavailability. The impact of exercise on flavanone bioavailability is potentially complex and requires further investigation. Cessation of training by physically active individuals provides a useful model to study the physiological effects of exercise (22), and the objective of this study was to determine the impact of a 7-day detraining period on the bioavailability of OJ (poly)phenols in endurance-trained male athletes.

SUBJECTS AND METHODS

Chemicals and materials

The chemicals used in the study and their sources were as described previously by Pereira-Caro et al. (17). Synthetic urine (Negative Urine Control) was purchased from Sigma-Aldrich, Madrid, Spain).

Participants

Eligible participants of this study were endurance trained men with body mass index (BMI) < 25 kg/m^2 and maximal oxygen consumption ($\dot{V}O_2max$) ≥ 51.0 ml/kg/min. They were recruited by advertisements and word of mouth in the campus of the University of Glasgow and in other public places. Participants were non-smokers, with stable weight for one month prior to study

enrolment, and were not on any medication, nutritional supplement or special diet. Before enrolling in the study, participants underwent a detailed health screen regarding participant's health to exclude chronic illness, eating disorders and history of gastrointestinal diseases which could interfere with the results of the study. All participants gave written informed consent. The Ethics Committee of the College of Medical, Veterinary and Life Sciences, Glasgow University approved the study which was registered at ClinicalTrials.gov (NCT02627547).

Screening procedures and cardiorespiratory fitness assessment

During the screening sessions $\dot{V}O_2max$ tests were carried out to ensure that participants had a high level of cardiorespiratory fitness for their age group and, thus, could be classified as endurance-trained athletes. A $\dot{V}O_2max \geq 51.0$ ml/kg/min, which refers to fitness excellence (23), was considered as the main inclusion criterion. Prior to this test participants completed Health Screening and Physical Activity Readiness Questionnaires and had their height (Seca, Leicester, UK), weight, body fat (TBF-300, TANITA, Cranlea, UK) and BMI calculated.

The $\dot{V}O_2max$ assessment involved a continuous incremental exercise test to volitional exhaustion and was performed at 20-21°C with a relative humidity of 30-40% (24). The test was conducted on either a motorized treadmill (PPS Med, Woodway, Germany) or a cycle-ergometer (HP Cosmos Cyclus 2 Record-trainer, Nussdorf-Traunstein, Germany), depending on the participant's type of training. Preceding the test, participants were fitted with a heart rate monitor (Polar Sports Tester, Polar Electro Oy, Kempele, Finland) and were advised to warm up. During the treadmill test the participants began with a warm up period of 6 min at a speed of 8 km/h. After the warm up phase, running speed was gradually increased by 1 km/h every minute until participants reached exhaustion. During the cycle ergometer test participants were asked

to choose a familiar and comfortable pedalling rate greater than 60 rpm and to maintain it throughout the test. The 4 min warm up period at 100 W was followed by gradual increases in power output of 25 W every min until 200 W was reached at which point power output was increased by 25 W every 2 min until participants reached volitional exhaustion. During tests collection of expired gas was initiated when a significant increase in ventilation and heart rate was achieved; our experience and judgement was used to determine when the subject would reach exhaustion. Verbal encouragement was given to participants throughout the test. Exhaustion was defined as the time at which the subjects were no longer able to maintain the prescribed running speed or pedalling rate. Expired gas samples were collected using the Douglas bag technique (25) and heart rate and rating of perceived exertion (26) were recorded during 2-3 final stages of the test. Expired gas samples were analysed for O_2% and CO_2% (4100 Gas Purity Analyzer, Servomex, UK) volume (Dry gas meter, Harvard, Kent, UK), and temperature. Barometric pressure was measured using a standard mercury barometer. Oxygen consumption (VO_2) values were derived using Haldane transformation (27). The $\dot{V}O_2$ value obtained during the last expired gas collection was taken as the $\dot{V}O_2$max value.

Study design

Each subject participated in two 24 h OJ feeding trials: one during a period of normal training, the other immediately following 1 week of an absence of training. Participants were requested to maintain their normal training program during the week leading up to the first OJ feeding trial (trained state), and to ensure that they trained on the preceding day. They were asked to refrain from any training prior to a second OJ feeding trial (detrained state). Participants weighed and recorded their dietary intake and were asked to follow a diet low in

(poly)phenolic compounds by avoiding fruits and vegetables, chocolate, nuts, high-fibre products, and beverages such as tea, coffee and fruit juices, as well as to abstain from consuming alcohol, for the 2 days prior to their first OJ feeding trial in the trained state and to replicate their diet during the 2 days prior to the feeding trial in the detrained state.

On the morning of the OJ feeding trials, participants reported to the metabolic suite between 0800 and 0900 h after a 12-h fast and brought their 12 h excreted overnight urine sample. Height, body mass and body fat were measured. Participants then consumed 500 mL of OJ (Tropicana "With Bits"), homogeneity of samples was ensured by mixing and freezing in bulk and, except for water intake to maintain adequate levels of hydration, no other food or drink was allowed for the next 4 h. Four hours after beginning of the trial participants were provided with a white roll with butter. After 8 h, participants were provided with a standard low (poly)phenol meal (a buttered white roll with ham and cheese and potato chips/crisps) after which they left the laboratory and returned home to sleep at home. They were instructed to continue the low (poly)phenol diet that evening and return to the laboratory the next morning to provide overnight urine. During the feeding trials participants collected all urine excreted over the following time periods: 0-5, 5-8, 8-10 and 10-24 h. Urine was collected into sealable flasks kept on ice. The total volume of each urine fraction was recorded and 2 mL aliquots were stored at –80°C prior to analysis.

Analysis of orange juice and urine by HPLC-HR-MS detection

Urine samples and (poly)phenols in OJ were analysed using the procedures described by Pereira-Caro et al. (17). Briefly, aliquots of urine and OJ were analysed using a Dionex Ultimate 3000 RS UHPLC system comprising a UHPLC pump, a PDA detector scanning from 200 to 600

nm, and an autosampler operating at 4°C (Thermo Scientific, San Jose, CA). Reverse phase separations were carried out using a 150 x 4.6 mm i.d. 5 µm 100Å C18 Kinetex column (Phenomenex, Macclesfield, UK) maintained at 40°C and eluted at a flow rate of 1.0 mL/min with a 45 min gradient of 3-50% of 0.1% acidic methanol in 0.1% aqueous formic acid. After passing through the flow cell of the PDA detector the column eluate was split and 0.2 mL/min directed to an Exactive™ Orbitrap mass spectrometer fitted with a heated electrospray ionization probe (Thermo Scientific) operating in negative ionization mode. Analyses were based on scanning from 100 to 1000 *m/z*, with in-source collision-induced dissociation at 25.0 eV. The capillary temperature was 300°C, the heater temperature was 150°C, the sheath gas and the auxillary gas flow rate were both 20 units, the sweep gas was 3 and the spray voltage was 3.00 kV. Data acquisition and processing were carried out using Xcalibur 3.0 software.

Identification and quantification of OJ (poly)phenols and their urinary metabolites was achieved as described previously (17). Analysis of flavanone metabolites and phenolic catabolites in urine was carried out by selecting the theoretical exact mass of the molecular ion by reference to 0.1-750 ng standard curves. A linear response was obtained for all the available standards, as checked by linear regression analysis (R^2>0.999). Limits of detection (ranging from 0.02 to 0.09 ng), limits of quantification (0.08-0.5 ng) and precision of the assay (as the coefficient of intra-assay variation, ranging from 1.8 to 4.9%) were considered acceptable allowing the quantification of metabolites. All reference compounds used for calibration curves were made up in synthetic urine. In absence of reference compounds, metabolites were quantified by reference to the calibration curve of a closely related parent compound (17).

Statistical analysis

Data were assessed for normality of distribution using Shapiro-Wilk test and revealed that data was not normally distributed. The comparisons of responses measured at different time points were made by Friedman's ANOVA followed by Wilcoxon post-hoc signed-rank test. Wilcoxon post-hoc signed-rank test was used to determine whether differences in total and relative excretion of flavanone metabolites and phenolic catabolites were significant between the trained and detrained states. Significance was accepted at the $P<0.05$ level and data are presented as mean values ± SE unless stated otherwise. Statistical analyses were performed using Statistica (version 10.0; StatSoft, Inc., Tulsa, OK) and Minitab (version 17.3.1; Minitab, Inc., State College, PA).

Standard deviations for mean concentration differences between trained and detrained states were 0.6 μmol, 3.8 μmol and 4.7 μmol for total naringenin, total hespertin and total flavonones metabolites respectively. Thus, with 10 participants, a minimum detected differences of 0.6 μmol for total naringenin, 2.6 μmol for total hespertin, and 3.4 μmol for total flavonone metabolites at specified power of 80% were significant at the 5% level.

RESULTS

Participants

Of 16 eligible participants, 3 individuals declined to take part in the study because of time commitments, and thus 13 participants were enrolled (see Supplemental Figure 1 under "Supplemental data" in the online issue). Of these 13 participants, one participant dropout before beginning the study due to illness. Of the 12 participants who completed study, two were excluded, as prior to the second feeding trial they did not follow low (poly)phenol diet. Thus, the study was completed by 10 endurance-trained men with a height of 178 ± 1.9 cm, a BMI of 21.7 ±

0.6 kg/m^2, percentage body fat of 7.5 ± 0.9% and a $\dot{V}O_2$max of 58.2 ± 1.7 mL/kg/min (mean values ± SE). The volunteers had been training on a routine basis for the past 4-12 years and typically performed 5-10 h of endurance training per week. They competed regularly in running events, such as marathons and half-marathons, at regional and national levels.

Identification and quantification of (poly)phenols in orange juice

The 500 mL of OJ consumed by the volunteers in both trials contained hesperetin-7-*O*-rutinoside (246 μmol), hesperetin-7-O-rutinoside-3′-*O*-glucoside (4 μmol), naringenin-7-*O*-rutinoside (62 μmol), 4'-*O*-methyl-naringenin-7-*O*-rutinoside (14 μmol), eriodictyol-7-*O*-rutinoside (4 μmol), apigenin-6,8-*C*-diglucoside (35 μmol), ferulic acid-4′-*O*-glucoside (16 μmol), coumaric acid-4′-glucoside (11 μmol), a sinapic acid-*O*-hexoside (6 μmol) and the amine *p*-sympatol (6 μmol) (**Table 1**). Thus, in total, the ingested juice contained 398 μmol of (poly)phenols of which 330 μmol were flavanones. The structures of the identified OJ component are presented in our earlier publication (17).

Excretion of flavanone metabolites in urine

As anticipated, no flavanone metabolites were detected in 0-24 h baseline urine collected prior to the consumption of 500 mL of OJ. Quantitative data on the urinary excretion of flavanone metabolites 0-5, 5-8, 8-10, 10-24 h after OJ intake by the 10 endurance-trained volunteers in both trained and detrained conditions are summarised in **Figure 1**. Hesperetin metabolites were excreted in urine in higher quantities than naringenin and eriodictyol metabolites. The 0-24 h excretion of hesperetin metabolites, and as a consequence the overall level of flavanones metabolites, was significantly higher after a 7-day break in training than the

quantities excreted during training. This was due to increased amounts of hesperetin metabolites excreted 5-8 h after OJ intake (6.1 ± 3.0 μmol compared to 2.5 ± 1.1 μmol). There were no statistically significant differences in excretion of naringenin and eriodictyol metabolites by subjects who consumed OJ in the trained and detrained condition (Figure 1).

The basis of the HPLC-HR-MS-based identifications of 19 flavanone metabolites was outlined in an earlier publication (17). The structures of the fully identified metabolites are illustrated in **Figure 2.** Quantitative estimates of the levels of the individual metabolites excreted 0-5, 5-8, 8-10, 10-24 and 0-24 h after OJ consumption by the trained and detrained volunteers are presented in Supplemental Table 1 in the on-line Supplemental Information. The 0-24 h data are summarised in **Table 2** which shows that urinary excretion of total flavanone metabolites was significantly higher in volunteers who consumed OJ after the detraining period compared to the trained trial (13.8 ± 8.2 μmol vs 17.2 ± 4.8 μmol). This was due to the significant increase in the levels of the main metabolites hesperetin-3′-*O*-glucuronide, hesperetin-7-*O*-glucuronide, hesperetin-3′-*O*-sulfate and a hesperetin-*O*-glucosyl-sulfate. There was no significant difference in excretion, in the trained and detrained states, of the lower quantities of naringenin and eriodictyl metabolites. The main naringenin metabolites were the 4′- and 7-*O*-glucuronides while trace amounts of an eriodictyol-sulfate and an eriodictyol-*O*-glucuronyl-sulfate were also excreted (Table 2).

The overall 0-24 h excretion in the trained and detrained states expressed as a percentage of intake was, respectively, 3.8% and 4.8% for hesperetin metabolites, 5.4% and 6.2% for naringenin metabolites and 5.0% and 5.0% for metabolites of eriodictyol metabolites (Table 2). Overall flavanones metabolite excretion was, respectively, 4.2% and 5.2% of intake for the trained and detrained stages of the study. These values are significantly different (Table 2).

243

244 **Urinary excretion of phenolic and aromatic catabolites**

245 Previously, 65 phenolic and aromatic catabolites were identified in urine after OJ
246 consumption by the trained and detrained volunteers (17). Supplemental Table 2 contains
247 estimates of the 33 phenolic compounds present in quantifiable amounts that were excreted in
248 urine 0-5 h, 5-8 h, 8-10 h and 10-24 h following OJ intake by the two groups of volunteers. For
249 the structures of these compounds see Supplemental Figure 2.

250 The various phenolics are not necessarily exclusively the products of colonic microbiota-
251 mediated degradation of the OJ (poly)phenols which reached the distal gastrointestinal tract
252 (GIT). A portion of the catabolites in Supplemental Table 2 are also products of endogenous
253 pathways unrelated to OJ intake (28). Hence, they were present to varying degrees in the 12 h
254 overnight urine collected prior to OJ intake after volunteers had been on a low (poly)phenol diet
255 low for 36-48 h. These 0-12 h baseline values were, therefore, used on a per hour basis to
256 subtract from the total amounts of phenolic and aromatic catabolites excreted in urine 0-24 h
257 post-supplementation in order to assess the impact of OJ consumption. The data are presented in
258 **Table 3**. A total of 202 ± 54 μmol were excreted over the 24 h period by the trained group, which
259 corresponds to 51% of the 398 μmol (poly)phenol intake, while overall phenolic catabolite
260 excretion in the detrained condition increased, but not significantly, to 236 ± 74 μmol, which is a
261 59% recovery.

262 Excretion of some of the 33 individual phenolic compounds did increase significantly after
263 OJ intake by both groups (Table 3). Three of these catabolites, namely 3-(3´-hydroxy-4´-
264 methoxyphenyl)hydracrylic acid, a methoxyphenylacetic acid-*O*-glucuronide and 3´-
265 hydroxyphenylacetic acid, were excreted in amounts after cessation of training that were

significantly higher than excretion prior to stopping training. These increases were relatively minor compared to the overall excretion of phenolic catabolites. However, the increased excretion of 3-(3´-hydroxy-4´-methoxyphenyl)hydracrylic acid, by both groups after OJ intake, is of interest as it has been proposed as a biomarker of hesperetin intake (14, 18).

Volunteer variations in excretion of flavanones metabolites and phenolic catabolites

Table 4 summarises data obtained with the individual volunteers on the total 0-24 h urinary recovery of metabolites from a 330 μmol intake of flavanones and the phenolic catabolite recovery from the ingested 398 μmol of (poly)phenolics. Detraining significantly increased mean flavanone metabolite excretion from 13.8 μmol to 17.2 μmol, with 9 of the 10 subjects showing increased excretion with detraining. Detraining increased mean phenolic catabolite excretion from 202 μmol to 236 μmol but this increase was not statistically significant although 8 of the individual volunteers did show an increase (Table 4).

There was noticeable variation between the volunteers which is reflected in the range of the amounts of metabolites and catabolites shown in Table 4. For instance, in the trained condition volunteers 1, 2 and 3 excreted 4.8-4.9 μmol of flavanones metabolites while volunteer 9 excreted 34 μmol and subject 10, 42 μmol. There was, however, a consistency in that low excreters in the trained condition were also low excreters when they stopped training for 7 days and likewise with the high excreters. For instance, volunteer 1, 2 and 3 excreted 4.8-4.9 μmol of flavanone metabolites when training and 6.7-7.5 μmol after stopping training. Subject 10 excreted 42 μmol of metabolites in the trained condition and 50 μmol after stopping training (Table 4). The trend was less evident with the higher level excretion of the phenolic catabolites. Four of 5 subjects who excreted >200 μmol of catabolites also excreted >200 μmol after stopping

289 training for 7 days (volunteers 1, 2, 5 and 6), the exception being volunteers 7. The one volunteer
290 who excreted <200 μmol of catabolites while training also did so after cessation of training
291 (volunteer 10) (Table 4).

292

293 **DISCUSSION**

294 In this study with endurance trained athletes flavanone bioavailability was assessed on the
295 basis of urinary excretion after OJ intake. Although plasma profiles can supply useful
296 information, unlike cumulative urinary excretion, they are not an accurate quantitative guide of
297 absorption because the presence of metabolites and catabolites in the circulatory system is
298 transient as they are rapidly removed from the bloodstream via renal excretion (18, 28, 29).

299 Our previous OJ feeding study showed that excretion of hesperetin and naringenin
300 metabolites 0-24 h after OJ consumption corresponded to 16% intake, while excretion of
301 phenolic catabolites was equivalent to ~88% of (poly)phenol intake (18). In the current
302 investigation, with a very different population of endurance trained male athletes, the ingestion
303 of OJ during training resulted in a lower level of excretion with 4.2% recovery of flavanone
304 metabolites in urine collected 0-24 h after intake (Table 2). Excretion of phenolic catabolites
305 during training was also reduced, but not to the same degree, with 51% of (poly)phenol intake
306 appearing in urine (Tables 3 and 4). Bioavailability was increased significantly, but none-the-
307 less marginally, when the athletes stopped training for 7 days at which point OJ consumption
308 resulted in a 5.2% excretion of flavanone phase II metabolites (Table 2). The overall excretion of
309 phenolic catabolites was substantially higher than that of the flavanone metabolites, and
310 increased from 51% to 59% of intake after cessation of training, but the increase is not
311 statistically different (Table 4).

312 The data in Table 2 indicates that hesperetin-3'-*O*-glucuronide, the urinary main flavanone
313 metabolite, is a good biomarker of hesperetin and OJ intake while the information in Table 3
314 confirms the earlier suggestion (14, 18) that 3-(3´-hydroxy-4´-methoxyphenyl)hydracrylic acid,
315 because of its 3′-hydroxy-4′-methoxy structure, is also a key indicator of hesperetin
316 consumption. Many other phenolic compounds were also excreted in increased quantities after
317 OJ consumption, most notably 4´-hydroxyphenylacetic acid (Table 3). However, most, if not all,
318 are colonic catabolites of other dietary (poly)phenols having been detected in feeding studies
319 with a number of products or following in vitro fecal incubations (30–41) and, thus, are not
320 specific indicators of flavanone intake.

321 The markedly lower levels of flavanone metabolite excretion by the endurance trained
322 volunteers compared to our previous study with less active subjects (4.2% vs 16% of intake)
323 could be due to a more rapid rate of gastrointestinal transport in the athletic subjects (20).
324 Detailed analysis of plasma pharmacokinetic profiles of (poly)phenol metabolites and catabolites
325 obtained with the current study will be the topic of a separate publication. However, the profiles
326 for hesperetin-7-*O*-glucuronide and ferulic acid-4'–sulfate shown in Figure 3 in the
327 Supplementary Information indicate that cessation of training for 7 days had no discernible
328 impact on gastrointestinal transport of flavanones, unlike co-ingestion of OJ with yogurt
329 demonstrated in an earlier study (42).

330 Endurance training has been reported to bring about changes in the colonic microbiota
331 (43) which, arguably, could limit microbiota-mediated cleavage of the rutinoside moiety of
332 flavanone glycosides and so reduce the amount of the hesperetin and naringenin released for
333 absorption in the distal GIT. In addition, training results in adverse physiologic adaptations in
334 the gut (44) and enhances muscle blood flow up to 12-16 h after the last exercise session (21).

These events could inhibit gut function and further reduce the absorption of the flavanone aglycones. While stopping training for 7 days significantly increased flavanone metabolite excretion from 4.2% to 5.2% of intake (Table 2) a longer period without training would appear to be required to attain the 16% excretion of flavanones metabolites observed when OJ was consumed by volunteers who were not endurance athletes (18).

The main flavanone metabolites, hesperetin-3'-*O*-glucuronide, hesperetin-7-*O*-glucuronide, hesperetin-3'-sulfate (Table 2), are absorbed principally in the colon after microbiota-mediated cleavage of the rutinose moiety of hesperetin-7-*O*-rutinoside (13, 45). These metabolites, at concentrations that can be achieved in vivo, have been reported to exert anti-atherogenic effects, via ameliorating monocyte adhesion to endothelial cells and modulating the expression of proteins associated with inflammation and supressing induced inflammation (46, 47). They also efficiently reduce the TNF-α-induced migration of human aortic endothelial cells. This was accompanyed and mediated by significant decreases in PAI-1 levels, a thrombogenic protein, which is involved in a wide range of cardiovascular diseases, as well as in the cell migration (48). The importance of the colonic microbiota is further emphasized by the fact that a number of the colon-derived phenolic catabolites which increased significantly after OJ intake (Table 3) also exert potential protective effects in ex vivo and in vitro test systems at physiological doses (49-52).

There are substantial inter-individual differences with, for instance, excretion of flavanones metabolites after cessation of training ranging from 6.2 μmol to 50 μmol (Table 4). Intra-individual differences, however, were much smaller with high excreters producing relatively high amounts of metabolites in both the trained state and after cessation of training (volunteers 9 and 10) while low excreters maintained this condition after stopping training (volunteers 1-3). The inter-individual variation in the absorption of OJ flavanones (53, 54), and in the

bioavailability of dietary (poly)phenols in general, is an area of increasing interest because information of variations in the capacity to metabolize these compounds may lead to a better understanding of the beneficial effects of plant bioactive compounds against diseases, particularly, their role in healthy ageing and cardiometabolic risk reduction (55).

The results of the current study contradict the findings of Medina et al. (19) who reported that after consumption of an Aronia-citrus juice, the quantity of flavanone metabolites excreted in urine by triathletes was 5-fold higher than excretion by control, more sedentary volunteers. The two groups of volunteers consumed a juice containing 80 mg of flavanones which, assuming they were mainly hesperetin- and naringenin–*O*-rutinosides, equates with a dose of ~130 μmol. The aglycones hesperetin and naringenin, released by enzyme hydrolysis of urine prior to analysis, were quantified by HPLC-MS. The 0-24 h post-consumption excretion was estimated to be ~0.3% of intake by the control group and ~1.3% by the triathletes. The excretion by the control group is very low compared with 16% obtained in our more recent acute feeding study with OJ (18). Although hydrolysis of flavonoid glucuronide and sulfate metabolites by mollusc enzymes, which have inconsistent titre, does not result in complete cleavage, especially of sulfates, and as a consequence under-estimates metabolite levels (56, 57), the efficiency of this step is unlikely to have been so low as to account for estimates of urinary excretion being ~50-fold lower than those detected by Pereira-Caro et al. (18, 58) and also markedly lower than flavanone excretion reported by other investigators (40, 53, 54, 59, 60).

CONCLUSIONS

Short duration cessation of physical training slightly, but significantly, enhanced the bioavailability of OJ polyphenols due to increased excretion of hesperetin metabolites. When

compared with data obtained in previous OJ feeding studies (18) the bioavailability of OJ flavanones in endurance trained male athletes was lower than in less active individuals. The more substantial excretion of colon-derived phenolic catabolites after OJ intake was not statistically different in the trained and detrained states but was lower than observed in the previous study with volunteers who were not involved in a training programme. To what extent long-term participation in endurance training and reduced flavanone bioavailability, and potentially lowered bioavailability of other dietary (poly)phenolics, impacts on health and ageing remains to be determined. Hesperetin-3'-*O*-glucuronide and 3-(3´-hydroxy-4´-methoxyphenyl)hydracrylic acid are urinary biomarkers of the consumption of hesperetin-containing OJ and other citrus products.

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Authors contributions

D.M. contributed to the design of the study, supervised the assessments of cardiorespiratory fitness the volunteers and the feeding study, conducted statistical analyses and drafted the manuscript and its revisions; A.L.G. contributed to the design of the study and the statistical analyses and the preparation of the manuscript; T.P and A.M.-N. carried out the assessment of fitness of the volunteers and the feeding study; G.P.-C. and I.A.L conducted the

HPLC-HR-MS analyses, contributed to the statistical analysis and drafting of the manuscript and its revisions; J.M.M.R contributed to the analytical aspects of the study and the drafting of the manuscript; A.C. assisted in the design the study, supported the HPLC-HR-MS analyses and drafted the manuscript and its revisions.

Conflict of interests

AC is a consultant for Mars, Inc. and has received unrestricted research grants from Mars and research grants from other food companies and government agencies with an interest in health and nutrition. The other authors declare no conflict of interest.

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TABLE 1

Quantities of (poly)phenols in 500 mL of orange juice[1]

Orange Juice (Poly)phenols	**µmol in 500 mL**
Hesperetin-7-*O*-rutinoside	246
Hesperetin-7-*O*-rutinoside-3′-*O*-glucoside	4
Naringenin-7-*O*-rutinoside	62
4′-*O*-methyl-naringenin-7-*O*-rutinoside	14
Eriodictyol-7-*O*-rutinoside	4
Total flavanones	*330*
Apigenin-6,8-*C*-diglucoside	35
Total flavonoids	*35*
Ferulic acid-4′-*O*-glucoside	16
Coumaric acid-4′-glucoside	11
Sinapic acid-*O*-hexoside	6
Total phenolic acids	*33*
p-Sympatol	6
Total amines	*6*
Total (Poly)phenols	**398**

[1] Data expressed as µmol, SEM <5% of the mean in all instances (n = 3).

TABLE 2

Urinary excretion of flavanone metabolites 0-24 h after the ingestion of 500 mL of orange juice in trained and detrained states [1-3]

Naringenin metabolites	**Trained**	**Detrained**
Naringenin-4′,7-*O*-diglucuronide	0.02 ± 0.01	0.02 ± 0.01
Naringenin-5,7-*O*-diglucuronide	0.10 ± 0.09	0.03 ± 0.02
Naringenin-4′,5-*O*-diglucuronide	0.09 ± 0.06	0.04 ± 0.03
Naringenin-*O*-glucuronyl-sulfate	0.02 ± 0.01	0.02 ± 0.01
Naringenin-4′-*O*-glucuronide	1.8 ± 1.0	2.1 ± 1.1
Naringenin-7-*O*-glucuronide	2.0 ± 1.0	2.3 ± 1.1
Naringenin-4′-sulfate	0.1 ± 0.1	0.2 ± 0.1
Total naringenin metabolites	**4.1 ± 2.2** ***(5.4%)***	**4.7 ± 2.4** ***(6.2%)***
Hesperetin metabolites	**Trained**	**Detrained**
Hesperetin-3′,7-*O*-diglucuronide	0.02 ± 0.02	0.02 ± 0.02
Hesperetin-5,7-*O*-diglucuronide	0.3 ± 0.3	0.3 ± 0.3
Hesperetin-3′,5-*O*-diglucuronide	0.3 ± 0.2	0.3 ± 0.2
Hesperetin-5-*O*-glucuronide	0.06 ± 0.03	0.06 ± 0.03
Hesperetin-7-*O*-glucuronide	0.8 ± 0.6	1.0 ± 0.6[3]
Hesperetin-3′-*O*-glucuronide	6.0 ± 3.6	7.6 ± 4.4[3]
Hesperetin-sulfate	0.2 ± 0.1	0.2 ± 0.2
Hesperetin-3′-*O*-sulfate	1.7 ± 1.0	2.4 ± 1.3[3]
Hesperetin-*O*-glucosyl-sulfate	0.08 ± 0.04	0.11 ± 0.06[3]
Total hesperetin metabolites	**9.4 ± 5.8** ***(3.8%)***	**12.0 ± 7.0** ***(4.8 %)***[3]
Eriodictyol metabolites	**Trained**	**Detrained**
Eriodictyol-sulfate	0.1 ± 0.1	0.1 ± 0.1
Eriodictyol-*O*-glucuronyl-sulfate	0.1 ± 0.1	0.08 ± 0.09
Total eriodictyol metabolites	**0.2 ± 0.2** ***(5.0%)***	**0.2 ± 0.2** ***(5.0%)***
Total flavanone metabolites	**13.8 ± 4.2** ***(4.2%)***	**17.2 ± 4.8** ***(4.8%)***[3]

[1] The orange juice contained 330 µmol of flavanones (76 µmol naringenin-*O*-glycosides, 250 µmol hesperetin-*O*-glycosides, 4 µmol eriodictyol-7-*O*-rutinoside).

[2] Data are presented in µmol as mean values ± SE (n=10) and in bold italics as a percentage of intake.

[3] Significant increase in the excretion by the detrained volunteers ($P<0.05$, Wilcoxon signed rank test).

TABLE 3

Quantities of the main phenolic and aromatic compounds excreted in urine collected for 12 h prior to supplementation (baseline) or 0-24 h after the ingestion of 500 mL of orange juice in trained and detrained states.[1–5]

	Trained		Detrained	
Phenolic catabolites	**Baseline[5]**	**Post-OJ intake**	**Baseline[5]**	**Post-OJ intake**
Cinnamic acids				
Coumaric acid-4′-*O*-sulfate	0.02 ± 0.02	0.1 ± 0.0	0.05 ± 0.05	0.15 ± 0.10
Caffeic acid-3′-sulfate	0.2 ± 0.1	< LOQ	0.2 ± 0.1	0.02 ± 0.01
Ferulic acid	0.03 ± 0.02	0.06 ± 0.02	0.02 ± 0.01	0.1 ± 0.1
Ferulic acid-4′-*O*-glucuronide	0.2 ± 0.05	0.7 ± 0.2	0.02 ± 0.01	1.1 ± 0.2[3]
Ferulic acid-4′-sulfate	2.2 ± 0.8	7.8 ± 2.5[3]	2.0 ± 1.0	5.9 ± 2.1[3]
Isoferulic acid-3′-*O*-glucuronide	0.06 ± 0.03	1.6 ± 0.5[3]	0.07 ± 0.05	1.3 ± 0.4[3]
Total cinnamic acids	**2.7 ± 1.1**	**10.3 ± 3.3[3]**	**2.3 ± 1.7**	**8.6 ± 2.9[3]**
Phenylhydracrylic acids				
3-(3′-Hydroxyphenyl)hydracrylic acid	1.3 ± 0.8	0.2 ± 0.3	1.3 ± 1.0	0.6 ± 0.2
3-(3′-Hydroxy-4′-methoxyphenyl)hydracrylic acid	0.3 ± 0.2	18.5 ± 5.8[3]	0.2 ± 0.2	21 ± 6[3,4]
Total phenylhydracrylic acids	**1.6 ± 1.0**	**18.7 ± 6.1[3]**	**1.5 ± 1.2**	**21.6 ± 6.2[3]**
Phenylpropionic acids				
3-(4′-Hydroxyphenyl)propionic acid-3′-*O*-sulfate	1 ± 1	0.5 ± 0.2	1 ± 1	0.9 ± 0.4
3-(3′-Methoxy-4′-hydroxyphenyl)propionic acid	0.3 ± 0.3	0.6 ± 0.2	0.10 ± 0.06	1.0 ± 0.3[3]
3-(3′-Methoxyphenyl)propionic acid-4′-*O*-glucuronide	0.6 ± 0.4	1.3 ± 0.4	0.5 ± 0.2	1.0± 0.4
3-(4′-Methoxyphenyl)propionic acid-3′-*O*-glucuronide	0.06 ± 0.05	6.3 ± 2.0[3]	0.06 ± 0.04	5.7 ± 1.8[3]
3-(3′-Methoxyphenyl)propionic acid-4′-*O*-sulfate	1.2 ± 0.7	2.6 ± 0.8	0.4 ± 0.2	2.5 ± 0.8[3]
3-(4′-Methoxyphenyl)propionic acid-3′-*O*-sulfate	0.06 ± 0.05	3.2 ± 1.0[3]	0.05 ± 0.04	3.3 ± 1.0[3]
3-(Phenyl)propionic acid	0.2 ± 0.2	0.9 ± 0.3[3]	0.5 ± 0.7	0.7 ± 0.2
Total phenylpropionic acids	**5.5 ± 3.7**	**15.4 ± 4.9[3]**	**4.4 ± 3.5**	**15.1 ± 4.9[3]**
Phenylacetic acids				
3′,4′-Dihydroxyphenylacetic acid	0.2 ± 0.1	1.1 ± 0.4[3]	0.4 ± 0.4	0.7 ± 0.3
Hydroxyphenylacetic acid-3′-sulfate	1.2 ± 0.5	< LOQ	0.7 ± 0.4	< LOQ
3′-Methoxy-4-hydroxyphenylacetic acid	0.8 ± 0.2	0.7 ± 0.2	0.6 ± 0.2	1.1 ± 0.1

Methoxyphenylacetic acid-*O*-glucuronide	0.8 ± 0.5	0.05 ± 0.01	0.3 ± 0.1	0.7 ± 0.3[3,4]
3´-Methoxyphenylacetic acid-4´-sulfate	1.2 ± 0.7	< LOQ	0.8 ± 0.5	0.2 ± 0.2
4´-Methoxyphenylacetic acid-3´-sulfate	0.9 ± 0.3	0.6 ± 0.2	0.7 ± 0.4	0.7 ± 0.3
3´-Hydroxyphenylacetic acid	0.3 ± 0.1	< LOQ	0.3 ± 0.3	1.7 ± 0.6[3,4]
4´-Hydroxyphenylacetic acid	6 ± 2	104 ± 24[3]	3 ± 1	122 ± 38[3]
Phenylacetic acid	0.7 ± 0.4	0.8 ± 0.2	0.4 ± 0.2	1.5 ± 0.4[3]
Total phenylacetic acids	**11.8 ± 4.7**	**107.2 ± 24[3]**	**7.4 ± 3.8**	**128.6 ± 40[3,4]**
Benzoic acids				
3-Hydroxybenzoic acid-4-sulfate	0.6 ± 0.3	4.8 ± 0.1[3]	0.4 ± 0.2	6.9 ± 0.1[3]
4-Hydroxybenzoic acid	0.3 ± 0.2	0.1 ± 0.1	0.2 ± 0.1	0.3 ± 0.1
Benzoic acid-4-sulfate	2 ± 1	< LOQ	1.6 ± 0.6	< LOQ
Total benzoic acids	**3.0 ± 1.5**	**4.9 ± 0.2**	**2.3 ± 1.0**	**7.2 ± 0.1[3]**
Mandelic acids				
3´-Methoxy-4´-hydroxymandelic acid	0.7 ± 0.2	0.35 ± 0.1	0.4 ± 0.1	0.9 ± 0.1
4´-Hydroxymandelic acid	0.6 ± 0.2	1.8 ± 0.6[3]	0.30 ± 0.07	2.4 ± 0.1[3]
Total mandelic acids	**1.8 ± 0.4**	**2.15 ± 0.7[3]**	**0.7 ± 0.2**	**3.3 ± 0.2[3]**
Benzene triols				
1,3,5-Trihydroxyphenol	20.0 ± 3.4	10.9 ± 4.8	14.5 ± 5.2	9.3 ± 4.9
Total benzene triols	**20.0 ± 3.4**	**10.9 ± 4.8**	**14.5 ± 5.2**	**9.3 ± 4.9**
Hippuric acids				
4´-Hydroxyhippuric acid	1.3 ± 0.4	4.5 ± 1.3[3]	1.0 ± 0.6	5.7 ± 1.8[3]
3´-Hydroxyhippuric acid	1.5 ± 1.0	0.9 ± 0.5	1.5 ± 1.2	1.5 ± 0.6
Hippuric acid	24.0 ± 6.0	26.7 ± 8.6	18 ± 5	35.1 ± 11.0[3]
Total hippuric acids	**26.8 ± 7.4**	**32.1 ± 10.4**	**20.4 ± 6.8**	**42.3 ± 13.6[3]**
Total phenolic catabolites	**71.7 ± 22.4**	**202 ± 54[3]** ***(51%)***	**52.4 ± 22.0**	**236 ± 74[3]** ***(59%)***

[1] Data are expressed in μmol as mean values ± SE (n=10). The orange juice contained 398 μmol of (poly)phenols.

[2] Italicised numbers in parentheses represent excretion of phenolic catabolites as a percentage of (poly)phenol intake.

[3] Statistically significant higher excretion above baseline after orange juce consumption (P<0.05, Wilcoxon signed-rank test) in trained or detrained states.

[4] Significantly higher excretion of phenolic acid catabolites after orange juce intake following cessation of training for 7 days compared to consumption while training ($p<0.05$, Wilcoxon signed-rank test)

[5] Phenolic content of baseline urine collected for 12 h prior to orange juice intake used, on an excretion per hour basis, to subtract from excretion values of trained and detrained states obtained 0-24 h after supplementation to estimate increases in the phenolic content attributable to orange juice intake.

<LOQ – value below the limit of quantification.

TABLE 4

Summary of the quantities of total flavanone metabolites and phenolic colonic catabolites excreted in urine 0-24 h after the consumption of 500 mL of orange juice by 10 endurance trained athletes in and 7-day detrained states. [1–4]

		Volunteers											
Volunteers	**Metabolites/catabolites**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**Mean ± SE**	**Range**
Trained	Flavanone metabolites	4.8	4.9	4.9	6.9	7.8	8.2	11	13	34	42	13.8 ± 4.2	4.8 – 42
	Phenolic catabolites	221	333	171	154	242	258	250	160	150	77	202 ± 54	77 – 333
	Total relative to intake (%)	**57**	**85**	**44**	**40**	**63**	**67**	**65**	**43**	**46**	**30**	**54 ± 17**	**30 – 85**
Detrained	Flavanones metabolites	6.9⇑	6.7⇑	7.5⇑	8.8⇑	14⇑	17⇑	6.2	15⇑	39⇑	50⇑	17.2 ± 4.8[3]	6.2 – 50
	Phenolic catabolites	291⇑	388⇑	138	247⇑	367⇑	261	124	231⇑	189⇑	124⇑	236 ± 74	124 – 388
	Total relative to intake (%)	**75**	**99**	**36**	**64**	**96**	**70**	**33**	**62**	**57**	**44**	**64 ± 20**	**33 – 96**

[1] The juice contained 398 μmol of (poly)phenols including 330 μmol of flavanone. Values for phenolic catabolites background subtracted.

[2] Data expressed in μmol and in bold as a percentage of intake.

[3] Significant increase in the excretion of mean total metabolites (n = 10) following cessation of training for 7 days compared to the trained state (P<0.05, Wilcoxon signed-rank test)

[4] ⇑ indicates an increase with individual subjects following 7 days cessation of training

Figure Legends

Figure 1. Excretion of flavanone metabolites 0-5 h, 5-8 h, 8-10 h , 10-24 h and 0-24 h after the ingestion of orange juice containing 330 μmol of flavanones (76 μmol naringenin-O-glycosides, 250 μmol hesperetin-O-glycosides, 4 μmol eriodictyol-7-O-rutinoside) by endurance-trained volunteers while training (trained-black bars) and after stopping training for 7 days (detrained-grey bars). Data expressed in μmol as mean values ± standard error (n= 10). *Significant increase in the excretion by the detrained volunteers ($P<0.05$, Wilcoxon post-hoc test after Friedman's ANOVA).

Figure 2. Structures of selected flavanone phase II metabolites and 3-(3´-hydroxy-4´-methoxyphenyl)hydracrylic acid detected in urine after the ingestion of orange juice.