Physical activity increases the bioavailability of flavanones after dietary aronia-citrus juice intake in triathletes

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Abstract

Control and triathlete volunteers (n = 8 and n = 15, respectively) were given 400 mL and 200 mL of aronia-citrus juice (AC-juice), respectively. The 24 h urine samples were hydrolysed to determine the flavanones concentration by UPLC-QqQ-MS/MS. The flavanones metabolites in both groups of volunteers were glucuronides, sulfates, and sulfo-glucuronides, and the total excretion of flavanones increased fivefold in the triathletes compared with the control volunteers. The increase of ninefold in the homoeriodictyol of triathletes compared to control volunteers may suggest the overactivation of the microbiota metabolism caused by physical exercise. No differences concerning the bioavailability were detected between men and women in controlboth groups. The AC-juice could provide synergistic effects on health due to the increase in the bioavailability of flavanones, avoiding the deleterious effects caused by the overdosage of nutritional supplements.

Keywords

Aronia-citrus juice, bioavailability, dietary intervention, flavanones, triathletes

Highlights

- Flavanones excretion increased five-fold in triathletes *versus* control volunteers
- Physical activity increases the bioavailability of flavanones
- Changes in flavanones metabolites in 24 h hydrolyzed urine samples
- The main flavanones metabolites were glucuronides, sulfates and sulfo-glucuronides

1. Introduction

In the past decade, a number of clinical trials based on dietary interventions have been performed to establish the bioefficiency of distinct subclasses of polyphenols (Kay, 2010). The applicability of polyphenols to the athlete's world and their health benefits in this concrete hallmark remains scarcely addressed (Trombold, Barnes, Critchley, & Coyle, 2010). Currently, sport medicine and training require additional efforts to improve the efficiency and results, taking into consideration athletes' health. In order to gain a further insight on the relationship between training, nutrition, and health, a variety of nutritional supplements have been developed to increase the physical outcome of the training programs regardless the natural option of fruit juices with bioactive components (Trombold, Barnes, Critchley, & Coyle, 2010).

Citrus juices are known for their high content in flavonoids, especially flavanones (Fls) (400-600 mg L^{-1}) (Gil-Izquierdo, Gil, & Ferreres, 2002; Gil-Izquierdo, Gil, Ferreres, & Tomás-Barberán, 2001). These compounds are mostly attached to rhamnoglucosides which need to be removed by the colon microflora to absorbed (Silberberg, Gil-Izquierdo, Combaret, Remesy, Scalbert, & Morand, 2006). Fls have shown a much permanent systemic level due to the enterohepatic cycle, which allows the re-excretion of metabolites, by bile and their reabsorption in the small intestine or colon, and therefore, a longer stay of them at physiological level (Manach, Morand, Gil-Izquierdo, Bouteloup-Demange, & Rémésy, 2003).

In recent years, research in this field has been focused on the augmentation of Fls bioavailability, by different mechanisms, in order to increase the health-promoting properties of citrus juices. The combination of aronia (*Aronia melanocarpa*) with citrus juices could provide synergistic effects of the Fls plus the anthocyanins, among other bioactive compounds (Habauzit, Sacco, Gil-Izquierdo, Trzeciakiewicz, Morand, Barron,

et al., 2011). However, as far as we are aware, the effect of physical activity on the bioavailability of the target compounds (Fls) from aronia-citrus juices (AC-juice) remains unknown.

The aim of the present study was to identify of the circulating Fls metabolites after the intake of AC-juice, and compare their bioavailability in triathletes with that found in control volunteers. Besides, the Fls excretion was also evaluated, during a week before and after AC-juice intake, in triathletes.

2. Methods and Materials

2.1. Chemicals

Naringenin, eriodictyol, homoeriodictyol, hesperetin, and isosakuranetin were purchased from Extrasynthèse (Genay, France). Hesperetin 7-O-glucuronide was synthesized in our lab according to the method described by Boumendjel and co-workers (2009). All LC-MS grade solvents were obtained from J.T. Baker (NJ, USA). Formic acid and chlorhydric acid were purchased from Panreac (Barcelona, Spain). The *β*-glucuronidase, type H2 from *Helix pomatia* was obtained from Sigma-Aldrich (MI, USA).

2.2. AC-juice

The juice composition is based on the mixture of citrus juice (95%) with 5% of *Aronia melanocarpa* juice. The content in nutrients and caloric supply of the AC-juice that consumed control volunteers and triathletes were summarized in Tables 1 and 2, respectively detailing the percentage of contribution of the juice to the total diet.

2.3. Calculation of the training loads in triathletes.

The quantification of training programs is addressed to evaluate their effects on

physiological adaptation and subsequent performance (Borresen & Lambert, 2009). Currently, mathematical models are suitable for the quantification of the training loads. Indeed, the training impulse proposed by Banister & Calvert (1980) and its subsequent revision are recognized as valid for the quantification of training efforts. However, the evolution of sports performance has led to specific models for some exercises (Lucia et al., 2006).

In our work, the training load quantification was performed using the 'Objective Load Scale' (ECOs) developed by Cejuela & Esteve-Lanao (2011). The training load that supports a triathlete is an indication of its performance level. The training loads developed by the triathletes in the present work were similar to those found in other studies of endurance athletes (Lucia et al., 2006; Rodríguez-Marroyo et al., 2003).

The previously-developed method used allowed the quantification of the training loads in the sport of triathlon (swim, bike, run, and transitions), which are determined by the difficulty to maintain technique, delayed muscle soreness, typical workout density, and energy cost of each separate sport. The values of daily and weekly trainings were determined and summarized to assess the training load (ECOs) of each volunteer, depending on their physical characteristics and the intensity of the training program (the ECO data presented in this work are the average of the individual ECOs of the thriathletes). Briefly, and from a general point of view, intensity is considered exponentially –not linearly– with the aim of leveling-off the total training stress for a given performance level. The volume is quantified by time and this allows a better comparison of different performance levels and terrain conditions (pavement, uneven laps) (Cejuela & Esteve-Lanao, 2011).

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126 *2.4. Study Design*

127 Two clinical assays were developed with two intervention groups, control
128 population (non-trained volunteers) (n=8, 4 women and 4 men) and triathletes (n=15, 5
129 women and 10 men). Both groups were non smokers and did not receive any medication
130 during the experimental procedure. Women were not in menstrual days during the
131 study. Control population consumed two glasses of juice while triathletes ingested a
132 glass of juice according to their planning supervised by the nutritionist and the training
133 load (ECO).

134 In the first assay, the bioavailability and metabolism were compared between a
135 control group of non-trained volunteers and the group developing a strenuous and
136 chronic exercise in order to know the influence of the sport performance on the nature,
137 occurrence and excretion of flavanone metabolites in urine. The control volunteers
138 followed a diet without polyphenol-based food and products derived and the only
139 source of flavonoids was that provided by the AC-juice. The diet for triathletes
140 developed by the nutritionist provided the balanced products for a proper contribution
141 of carbohydrates, proteins, fats, vitamins and microelements where the plant-foods
142 (included those content flavanones) were ingested when were required. In detail, control
143 volunteers and triathletes (2500 ECOs of training load (Cejuela Anta & Esteve-Lanao,
144 2011) consumed a diet with equal intake of Fls provided by juice (86.1 mg) and by
145 juice+diet (88.1 mg), respectively for a week (Tables 1 and 2).

146 In the other assay, the objective was to know the evolution of the bioavailability and
147 metabolism of flavanones between a low charge of training and a period of strenuous
148 chronic exercise during two weeks. The triathletes followed during the first week a
149 control diet containing 45.0 mg of Fls (2400 ECOs) and 88.1 mg of Fls (provided by a

150 glass of juice+diet) (2500 ECOs) during the second week (Table 2). The study was
151 approved by the Bioethics Committee of University Hospital of Murcia and all
152 participants gave the written informed consent to participate in the dietary intervention
153 study.

154 Volunteer's urines were collected during 24h starting from 8.00 a.m are the intake
155 of the juice up to the 8.00 a.m. of the following day. They were immediately frozen at -
156 80 ºC until their analysis. Fls concentration was determined after total hydrolysis of
157 urine with urine *β*-glucuronidase (sulfatase activity) (Habauzit, et al., 2011; Manach,
158 Morand, Gil-Izquierdo, Bouteloup-Demange, & Rémésy, 2003).

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160 *2.5. UPLC-QqQ-MS/MS analysis*

161 The Fls metabolites in urines were analysed using an UPLC-MS/MS (Agilent
162 Technol., Germany) with an ACQUITY BEH C18 column (2.1 x 150 mm; 1.7 µm;
163 Waters, Ireland). The solvents were water/formic acid (99.9:0.1, *v/v*) (A) and
164 acetonitrile/formic acid (99.9:0.1, *v/v*) (B). The flow rate was 0.32 mL min^{-1} using a
165 linear gradient (t; %B): (0.00; 10), (3.50; 30), (8.00; 35), (8.01; 60), (10.00; 60), (10.01;
166 100), (12.00; 100), (12.10; 10). The volume injection was 10 µL. For the qualitative
167 analyses, the flavanone conjugates were analysed according the MRM transition
168 detailed in the Table 3 in order to know the original metabolites excreted in 24h-urine.
169 For the quantitation of the total flavanones in urine, the capillary exit voltage and
170 collision energy were previously optimized for the aglycones and hesperetin 7-*O*-
171 glucuronide. The MRM transitions (negative mode) for maximum intensity after
172 optimization of the MS parameters were at *m/z* 271→119, 301→151, 287→151,
173 301→151, and 285→164 for naringenin, hesperetin, eriodictyol, homoeriodictyol, and
174 isosakuranetin, respectively (Table 3). The product ion scan allowed the identification

of aglycones by comparison of the MS2 spectra with those corresponding standards. The urinary concentration of Fls was calculated on freshly and daily prepared standard curves using MassHunter software version B.04.00 (Agilent Technol., Germany).

2.6. Statistics

Quantitative data are presented as mean ± SD. Specific differences among the target compounds in samples from control volunteers and triathletes were examined by paired *t*-tests. Statistical analysis was conducted using SPSS 17.0 software package (LEAD Technol. Inc., USA), and the level of statistical significance was set at $P < 0.05$.

3. Results and discussion

3.1. Results

3.1.1 Qualitative analyses

The nature of the excreted metabolites was determined in 24-h urine samples after ingestion of 200 mL and 400 mL in triathletes and control volunteers, respectively. The goal of this assay was to know if there are differences between the urinary profile of the flavanone metabolites in both groups. The characterization of metabolites from total urinary Fls in non-hydrolyzed urine samples are presented in Table 3. The analysis of the non-hydrolyzed samples showed the occurrence of free aglycones as previous reports (Habauzit, et al., 2011; Manach, Morand, Gil-Izquierdo, Bouteloup-Demange, & Rémésy, 2003). Unparallely to the other metabolites, the sulfates of isosakuranetin and diglucuronides of eriodictyol were not detected, whereas homoeriodictyol was only found as aglycone form and conjugated to two glucuronides (Table 3). Hesperetin and naringenin showed the highest variety of conjugates including glucuronides, diglucuronides, sulphates, and sulfo-glucuronides. In addition, we distinguished a 7-*O*-glucuronide of hesperetin by comparison with its authentic marker.

Fls metabolites showed different retention times although had the same *m/z* $[M-H]^-$ and MS2 fragmentation patterns undergoing a 176 amu and 80 amu losses corresponding to a glucuronide acid and sulfate moieties, respectively (Habauzit, et al., 2011). There were no differences between the patterns of Fls metabolites excreted in urine of control *vs.* triathletes after AC-juice intake.

3.1.2 Quantitative analyses

In urine, the quantification of the Fls bioavailability has been carried out after enzymatic urine hydrolysis (Habauzit, et al., 2011). The total excretion of Fls was ~5-folds higher in triathletes compared with control volunteers (1860 ± 838 and 376 ± 122 nmol 24 h^{-1}, respectively) (Fig. 1A). This is of quite importance because of the amount of juice ingested by athletes was half than that consumed by control volunteers (200 and 400 mL, respectively). Then and according to this fact, the theoretical biovailability would be 10-fold higher respect to the ingested dose. In addition, when comparing control volunteers with triathletes, naringenin, eriodictyol, and hesperetin were the Fls that contributed more significantly to the increase of the total urinary Fls (~92%, ~88%, and ~53%, respectively) (Fig. 1B). The other two aglycones considered, homoeriodictyol and isosakuranetin, even though were at the lowest concentration among all considered Fls, presented a significant increase in bioavailability, between almost seven and nine-fold, respectively compared to that of control volunteers (theoretical 14 and 18-fold higher respect to the ingested dose, respectively) (Fig. 1B). Besides, no differences concerning to the bioavailability were detected in men and women in control and thriathletes volunteers.

In the second clinical trial, the triathletes developing elite training during 2 weeks, (control week (1^{st}): 2400 ECOs and AC-juice intake (200 mL) week (2^{nd}): 2500 ECOs) the differences on total Fls bioavailability between the first and second week were not

so clear since no significant differences were found (Fig. 2A), whereas eriodictyol and hesperetin excretion increased thanks to the additional flavanones intake provided by the AC-juice (Fig. 2B).

3.2 Discussion

Flavanones have been reported to be excreted conjugated to distinct combinations of glucuronic acid and/or sulfate in human urine (Manach, Morand, Gil-Izquierdo, Bouteloup-Demange, & Rémésy, 2003). The metabolites of hesperetin and naringenin were coincident with the glucuronide, diglucuronide, and sulfoglucuronide conjugates identified by Mullen and colleagues in urine (Mullen, Archeveque, Edwards, Matsumoto, & Crozier, 2008). Our qualitative analysis on the excretion of Fls after the intake of AC-juice by triathletes and control volunteers was consistent with the detection of the sulfates of eriodictyol, naringenin, and hesperetin (Table 3) as previously described (Bredsdorff, Nielsen, Rasmussen, Cornett, Barron, Bouisset, et al., 2010). The high abundance of sulfo-glucuronides compared to the sulfates may be due to the activation of the enterohepatic cycle which favors the longer stay of the metabolites in the human´s body (Manach, Morand, Gil-Izquierdo, Bouteloup-Demange, & Rémésy, 2003).

The first assay (control volunteers *vs* triathletes) showed that the total excretion of Fls increased five-fold in the triathletes compared with control volunteers. The absorption of Fls could be affected by a plethora of physiological factors linked to the physical activity. Among them, the triathletes may exhibit an increased intestinal motility secondary to the physical work. This entails an accelerated colorectal transit (Lippi, Banfi, Luca Salvagno, Montagnana, Franchini, & Cesare Guidi, 2008), and the modification of the bioavailability of dietary bioactive compounds, including Fls since the triathletes consumed the AC-juice 15 min after the training. During the exercise,

there is an increase in sympathetic and decrease in parasympathetic activity, which has a large influence on digestion because of their role in the innervation of the muscular layer within the intestine wall. Blood is usually moved away from the digestive tract in order to increase blood flow to the skeletal muscles and skin (Rao, Beaty, Chamberlain, Lambert, & Gisolfi, 1999) and the reduced blood supply may further inhibit gut function. However, after exercise, when blood flow is restored, the AC-juice was ingested by triathletes. At this point, the physiological changes may reestablish colonic motility allowing maximum exposure and absorption of nutrients including polyphenols and thus, the increase the Fls bioavailability. This augmented motility could favor the interaction of the AC-juice and food components of the diet with the gut microbiota.

A key point on Fls bioavailability is the cleavage of the original rhamnoglucosides forms of Fls into their aglycone, by intestinal enzymes of bacteria origin (α-rhamnosidases and β-glucosidases) (Erlund, Meririnne, Alfthan, & Aro, 2001). In fact, the interindividual variations, in the bioavailability of Fls, could be caused by differences in gut microflora. This leads us to think that elite training, as the triathlon, could modify the activity of the colon microbiota and therefore, the concentration of hydrolytic enzymes available, which might increase the Fls bioavailability. In this way, homoeriodictyol and its metabolites are produced as result of the action of the gut microbiota (Habauzit, et al., 2011). Thus, they could be valuable 'signalling compounds' about the overactivation of the microbiota metabolism since they were produced in higher extent in triathletes compared to control volunteers (9-fold higher).

In the second assay, the AC-juice intake contributed to an increase of Fls bioavailability in a normal diet adjusted for an elite training of triathletes. However, the two-fold higher intake of Fls provided by the AC-juice (88.1 mg) compared to the control diet (45 mg) was not proportionally delivered in the same extent concerning to

the Fls excretion rates. This fact would indicate to a saturation point of flavanone uptake caused by the physical activity. Taking into account the first and second assay, the high rate of Fls bioavailability in triathletes after the consumption of a glass of AC-juice compared to control volunteers was only owing to the physical activity regardless the origin of the Fls source in the diet (AC-juice or control diet) since in previous studies, this bioavailability has not been increased by a dietary dose of any type of citrus product consumed in a hallmark of a normal diet (Manach, Morand, Gil-Izquierdo, Bouteloup-Demange, & Rémésy, 2003).

4. Conclusions

In summary, the supplementation with a natural fruit juice may provide protection to the triathletes avoiding the intake of nutritional supplements, to enhance sport performance, and that eventually may entail adverse health outcomes. The total excretion of Fls increased five-fold in the triathletes compared with control volunteers (theoretical 10-fold higher respect to the ingested dose). It is of quite importance to underline the increase of 9-fold of the homoeriodictiyol in thriatletes compared to control volunteers (theoretical 18-fold higher respect to the ingested dose) since it could be considered as 'signalling compound' about the overactivation of the microbiota metabolism caused by physical exercise. Therefore, the effect of physical activity in triathletes consuming 1 glass of juice was enough to widely overcome the bioavailability of Fls generated by control volunteers after the intake of 2 glasses of juice. Despite the high intensity of the elite training by triathletes, a glass of AC-juice was able to increase the bioavailability of Fls in a diet already containing food products able to produce Fls metabolites at systemic level.

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Figure Captions

Fig. 1 Content of total (A) and single Flavanones (naringenin, eriodictyol, hesperetin, isosakuranetin, and homoeriodictyol) (B) (nmol $24h^{-1}$) after enzymatic hydrolysis determined in the 24h-urine of control volunteers (n=8) and triathletes (n=15). Bars with asterisks are statistically different at $*P<0.05$ and $***P<0.001$.

Fig. 2 Content of total (A) and single Flavanones (naringenin, eriodictyol, hesperetin, isosakuranetin, and homoeriodictyol) (B) (nmol $24h^{-1}$) after enzymatic hydrolysis determined in the 24h-urine of triathletes (n=15) before and after intake the AC-juice. Bars with asterisks are statistically different at $*P<0.05$, and $**P<0.01$.

393 **TABLES**

Table 1 Dietary characteristics and caloric intake of the control volunteers group (% of contribution of the juice to the diet has been detailed between brackets).

	Daily intake	AC-juice intake (400 mL)
Carbohydrates (g)	214.0	36.0 (16.8%)
Sugars (g)	38.1	13.3 (34.9%)
Proteins (g)	129.8	1.8 (1.4%)
Fats (g)	55.7	0.1 (0.2%)
Iron (mg)	12.5	0.5 (4.2%)
Vitamin C (mg)	162.4	154.8 (95.3%)
Vitamin E (mg)	5.0	0.2 (3.0%)
Vitamin A (µg)	220.1	3.0 (1.4%)
Total polyphenols (mg)	115.5	115.5 (100.0%)
Flavanones (mg)	86.1	86.1 (100.0%)
Flavones (mg)	29.4	29.4 (100.0%)
Energy intake (kcal)	1857.9	152.0 (8.2%)

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Table 2 Dietary characteristics, caloric intake, and training loads of the triathlete volunteers group (% of contribution of the juice to the diet has been detailed between brackets).

	Diet control	Diet + juice intake	AC-juice intake (200 mL)
Carbohydrates (g)	326.1	344.1	18.0 (5.2%)
Sugars (g)	121.3	127.9	6.6 (5.2%)
Proteins (g)	133.7	134.6	0.9 (0.6%)
Fats (g)	113.7	113.7	0.1 (0.1%)
Iron (mg)	20.9	21.2	0.3 (1.2%)
Vitamin C (mg)	178.7	256.1	77.4 (30.2%)
Vitamin E (mg)	21.0	21.1	0.1 (0.4%)
Vitamin A (µg)	2970.0	2971.5	1.5 (0.1%)
Total polyphenols (mg)	64.6	122.4	57.8 (47.2%)
Flavanones (mg)	45.0	88.1	43.1 (48.9%)
Flavones (mg)	0.7	15.4	14.7 (95.5%)
Flavan-3-ols (mg)	8.4	8.4	-
Flavonols (mg)	8.9	8.9	-
Isoflavones (mg)	1.6	1.6	-
Energy intake (kcal)	2820.0	2896.0	76.0 (2.6%)
Training loads			
ECOs	2400	2500	

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Table 3 Qualitative analysis of Fls metabolites non-hydrolyzed urine after the ingestion of AC-juice.

Retention time (min)	MRM transitions (*m/z*)	Compounds
Naringenin metabolites		
2.48	623→271	Naringenin-diglucuronide
3.15	623→271	Naringenin-diglucuronide
3.58	527→271	Naringenin-sulfoglucuronide
3.74	623→271	Naringenin-diglucuronide
3.74	527→271	Naringenin-sulfoglucuronide
4.53	447→271	Naringenin-glucuronide
4.64	447→271	Naringenin-glucuronide
4.69	351→271	Naringenin-sulfate
7.27	271→119	Naringenin
Hesperetin metabolites		
2.96	653→301	Hesperetin-diglucuronide
3.52	557→301	Hesperetin-sulfoglucuronide
3.68	653→301	Hesperetin-diglucuronide
3.82	557→301	Hesperetin-sulfoglucuronide
4.16	653→301	Hesperetin-diglucuronide
4.66	477→301	Hesperetin-glucuronide
4.88	477→301	Hesperetin-7-*O*-glucuronide
5.19	381→301	Hesperetin-sulfate
7.91	301→151	Hesperetin
Isosakuranetin metabolites		
4.09	541→285	Isosakuranetin-sulfoglucuronide
4.28	637→285	Isosakuranetin-diglucuronide
6.89	461→285	Isosakuranetin-glucuronide
9.79	285→164	Isosakuranetin
Eriodictyol metabolites		
2.09	463→287	Eriodictyol-glucluronide
3.02	367→287	Eriodictyol-sulfate
3.33	367→287	Eriodictyol-sulfate
3.65	543→287	Eriodictyol-sulfoglucuronide
5.77	287→151	Eriodictyol
Homoeriodictyol metabolites		
3.73	477→301	Homoeriodictyol-glucuronide
4.11	477→301	Homoeriodictyol-glucuronide
7.55	301→151	Homoeriodictyol

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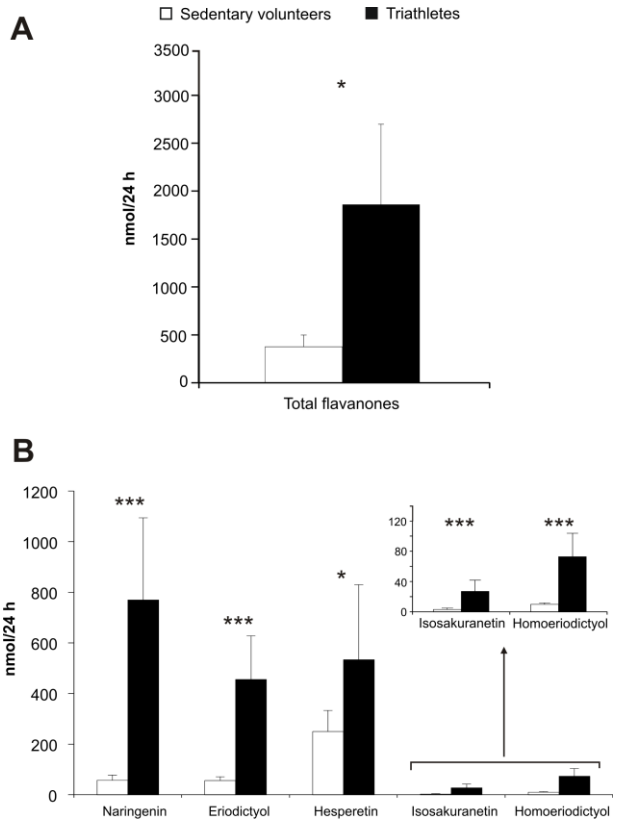
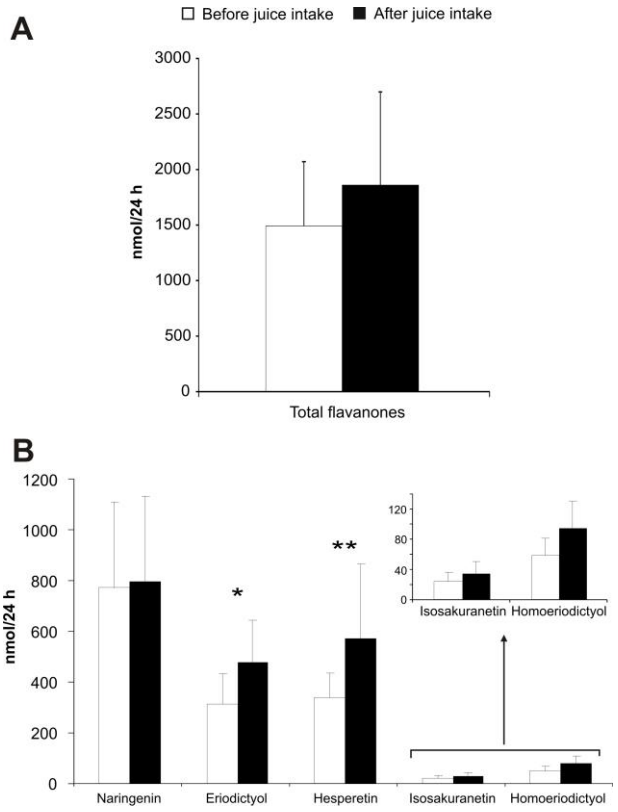
Figure 1.


A
Sedentary volunteers
Triathletes
nmol/24 h
3500
3000
2500
2000
1500
1000
500
0
*
Total flavanones
B
nmol/24 h
1200
1000
800
600
400
200
0
***
***
*
Naringenin
Eriodictyol
Hesperetin
120
80
40
0
***
***
Isosakuranetin
Homoeriodictyol
Isosakuranetin
Homoeriodictyol

413 **Figure 2.**



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