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## Bioavailability and antioxidant effects of orange juice components in humans

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### Abstract

Seven healthy females and six males consumed daily 256 mg vitamin C, 229 mg hesperidin (main flavonoid occurring as glycoside), 6 mg carotenoids (mainly luteins and cryptoxanthins), and 0.16 mg folate by incorporation of daily 236 mL of not-from-concentrate orange juice (OJ) into their habitual diet. At the end of three weeks mean vitamin C, folate, carotenoid, and flavanone plasma concentrations increased significantly relative to baseline by 59% ( $p < 0.001$ ), 46% ( $p = 0.018$ ), and 22% ( $p < 0.001$ ), and 8 fold ( $p = 0.045$ ), respectively. Flavanones were excreted in urine 9 fold more at the end of the intervention ( $p = 0.01$ ) but returned to baseline two days after study completion. After the 3-week intervention plasma concentrations of vitamins A and E did not change. 8-Hydroxy deoxyguanosine (8OHdG) in white blood cells declined by 16% ( $p = 0.38$ ;  $n = 11$ ), and in individuals with high baseline concentrations by 29% ( $p = 0.36$ ;  $n = 7$ ), respectively. LDL-/HDL-cholesterol ratio decreased but cholesterol (HDL, LDL, total) and thiobarbituric acid reactive substance plasma concentrations did not change significantly. We conclude from this pilot study that OJ is an excellent food source to enhance circulating concentrations of valuable hydrophilic as well as lipophilic phytochemicals.

### Keywords

orange juice; antioxidants; vitamins; flavonoids; folate; carotenoids

Oxidative damage is involved in many chronic diseases including the major causes of death in Western societies such as cardiovascular disorders and cancer (1). Oxidized LDL contributes to the formation of atherosclerotic lesions and poses an additional oxidant stress that injures smooth muscle and endothelial cells (2). Adiposity leads to oxidant stress because intracellular triglycerides cause increased superoxide formation (3) and stimulate adipocytes or preadipocytes to produce inflammatory cytokines (4) which induce formation of various oxidative radicals (5). Mutagens and carcinogens may act through the generation of free radicals which initiate a series of degenerative processes related to cancer, heart disease, and aging (6). Antioxidants may prevent these degenerative processes by various mechanisms including scavenging of free radicals. Intake as well as systemic levels of antioxidant micronutrients are associated with preventing cancer and heart disease in prospective epidemiologic as well as intervention studies when the exposure occurred through the diet (7-16). Antioxidants, markers of oxidative damage, and other risk factors like homocysteine, triglycerides, and LDL-cholesterol are therefore widely accepted surrogate means to assess risk for heart disease and cancer (17-19).

Orange juice (OJ) contains an array of potent antioxidants including flavonoids (hesperetin and naringenin predominantly as glycosides), carotenoids (xanthophylls, cryptoxanthins, carotenes), and vitamin C in addition to other beneficial phytochemicals, such as folate. All of these are believed to be significant contributors to the preventive effects of fruits and vegetables against cancer and heart disease (7,20).

A limited number of reports exists on the bioavailability of antioxidant micronutrients in humans associated with an assessment of risk markers as a result of OJ intake. After 4 weeks of the highest OJ dose (750 mL daily), but not at lower doses, circulating vitamin C, folate, HDL- cholesterol, and triacylglycerol levels were elevated without affecting homocysteine concentrations as assessed in 25 hypercholesterolemic subjects (21). After 2 weeks of daily 500 mL commercial fresh-squeezed OJ a significant inverse correlations between the plasma concentration of vitamin C and the isoprostane 8-*epi*-PGF<sub>2α</sub>, a marker for oxidative damage to lipids, was reported from 12 subjects (22). After 2 weeks of daily 237 or 472 mL OJ or supplemental vitamin C equivalent to the lower OJ dose were equally effective at increasing circulating vitamin C concentrations and at reducing plasma lipid peroxidation as measured by thiobarbituric reactive substances (TBARS) in 11 healthy adult women (23). Hesperetin and naringenin glycosides were found to be absorbed and eliminated very fast in humans consuming OJ and were suggested to contribute significantly to the pool of total plasma polyphenols known to be potent antioxidants (24,25).

The objective of the present study was to determine the effect of orange juice consumption on the antioxidant status and risk markers including the measurement of circulating flavonoids, carotenoids, vitamins A, C and E, folate, blood lipid profile, 8-hydroxy-deoxyguanosin (8OH-dG), TBARS and homocysteine in thirteen healthy human subjects of both genders.

## MATERIALS AND METHODS

### Materials

HPLC analyses with diode array detection were carried out on a model 'Karat' chromatography system including a binary pump model 125, an auto sampler model 507, and a multiple channel diode-array detector model 168 (all units from Beckman; Fullerton, CA). Liquid chromatography photo-diode array electrospray ionization mass spectrometry (LC/PDA/ESI-MS) was carried out with a multiple channel diode-array detector and a quadrupole ion trap mass spectrometer model LCQ Classic (ThermoElectron Corp., San Jose, CA). 8-Hydroxydeoxyguanosine was analyzed on an Agilent Technologies 1100 binary pump, autosampler, variable wavelength detector (Agilent Technology, San Diego, CA), and an ESA Coulochem II electrochemical detector (ESA, Bedford, MA). Absorbance readings were obtained from a DU-62 spectrophotometer (Beckman, Fullerton, CA). All solvents used for HPLC and absorbance readings were analytical grade or HPLC grade from Fisher Scientific (Fair Lawn, NJ). Butylated hydroxytoluene (BHT), hesperetin, hesperidin, naringenin, naringin, and all other chemicals were purchased from Sigma Chemicals Co. (St. Louis, MO).

### Subjects and Intervention Protocol

Thirteen healthy subjects, between 28–51 years of age (7 females and 6 males), of normal height and weight, nonsmoking, not on any medication including hormones or dietary supplements, without particular dietary patterns (e.g., vegetarians) consumed daily three 8 ounces of chilled not-from-concentrate orange juice (Tropicana Products, Inc., Bradenton, FL) in addition to their usual diet, which subjects were recommended not to change during the intervention. All procedures of the protocol were approved by the Institutional Review Board (Committee on Human Subjects) of the University of Hawaii. Before entering the study, all participants were informed about the protocol and signed the informed consent agreement.

## Collection of plasma and urine samples

On the morning before starting the intervention (baseline), after week 1 (day 8 of the intervention), and after completion of the intervention (day 22 of the study) a blood sample after fasting for at least 8 hours was drawn at 8 a.m. by venipuncture, and overnight urine was collected. Blood was collected under subdued light into Na-heparin vacutainers followed by immediate processing (plasma, white blood cells) in the dark at  $<8^{\circ}\text{C}$  to avoid analyte degradation. Urine was weighed and stored aliquoted at  $-70^{\circ}\text{C}$ .

## Analysis of phytochemicals in orange juice, plasma, and urine

Carotenoid concentrations in juice were determined slightly modified from previous protocols (26-28) by applying our previously established HPLC system (29). Final values were obtained from peak areas using calibration curves of solutions of authentic standards and by adjustment to internal standard recovery. Inter-assay coefficients of variation (cv) for beta-carotene, and beta-cryptoxanthin were 9% and 8%, respectively, when analyzed from orange juice. Plasma carotenoids, retinoids, and tocopherols were analyzed with the same HPLC system after extraction from plasma as reported previously (29). Beta and gamma tocopherol were detected together because these two compounds coelute in reverse-phase chromatography (30). Quality of analysis was assured by participation in round robin tests organized by the National Institute for Standards and Technologies (NIST, Gaithersburg, MD). Accuracy for the lipid soluble vitamin assay was assured by participation in a quality assurance program organized by the National Institute for Standards and Technologies (NIST), Gaithersburg, MD. Our results for the main serum analytes did not exceed more than 1 standard deviation from the assigned value. Precision of tr LUT/ZEA, cis LUT/ZEA, tr AH-LUT, cis AH-LUT, a-CRX, tr b-CRX, cis b-CRX, LYC, DHLYC, a-CAR, tr b-CAR, cis b-CAR, total carotenoids, d-TOC, b+g-TOC, a-TOC, and retinol was examined by an external plasma sample run every second batch (a total of 9 runs) and revealed the following cv at the given mean concentrations: 7% at  $219\ \mu\text{g/L}$ , 9% at  $99\ \mu\text{g/L}$ , 4%  $62\ \mu\text{g/L}$ , 8% at  $37\ \mu\text{g/L}$ , 9% at  $32\ \mu\text{g/L}$ , 7% at  $56\ \mu\text{g/L}$ , 13% at  $30\ \mu\text{g/L}$ , 8% at  $195\ \mu\text{g/L}$ , 17% at  $47\ \mu\text{g/L}$ , 7% at  $25\ \mu\text{g/L}$ , 10% at  $138\ \mu\text{g/L}$ , 11% at  $17\ \mu\text{g/L}$ , 4% at  $909\ \mu\text{g/L}$ , 17% at  $685\ \mu\text{g/L}$ , 8% at  $515\ \mu\text{g/L}$ , 5% at  $11387\ \mu\text{g/L}$ , and 5% at  $413\ \mu\text{g/L}$ .

Vitamin C content in the orange juice was analyzed by reversed phase HPLC with UV detection (31). Inter-assay variation was found to be 4.8% using orange juice. Vitamin C concentrations in plasma were analyzed colorimetrically using dichlorophenolindophenol (32). Quality of analysis was assured by participation in round robin tests organized by NIST. Accuracy of this assay was again assured by participation in a quality assurance program from NIST and revealed deviations from the assigned value of not more than 7% at low concentrations ranging from 18–24  $\mu\text{M}$ . Folate concentrations in the orange juice and in plasma were analyzed using the Bio-Rad Quantaphase II radioassay kit according to the instruction manual (Bio-Rad Laboratories, Inc., Hercules, CA). Lymphocheck serum controls I, II and III were run with each batch of 40 samples. All samples and controls were run in duplicates. If cv was  $\geq 10\%$  samples were reanalyzed. The intra- and inter-assay cv was 3.5% and 4.9% at 4.8 nM, 8.5% and 9.4% at 11.6 nM, and  $<1\%$  and 12.4% at 25.1 nM, respectively. of flavonoids and their glycoside from orange juice were determined by RP-HPLC with diode-array detection without hydrolysis as described in detail recently (33). Flavonoid concentrations from plasma and urine were analyzed by LC/ESI-MS after enzymatic hydrolysis of glucuronide and sulfate conjugates to their respective aglycones (hesperetin, naringenin) as described previously (34). Detection limits were found to be 0.1–5 nmol/L depending on the analyte and inter- assay cv coefficients of variations 8–22% at levels below 20 nmol/L, 7–14% at levels 20 nmol/L – 100 nmol/L, and 3–12% at levels over 100 nmol/L.

Spiking recovery from plasma for naringenin and hesperetin were found at 80% and 84%, respectively; inter-assay cv were 8% (80.0 nM) and 6% (99.7 nM), respectively.

Triacylglycerol (TAG), HDL and total cholesterol concentrations in plasma were determined by colorimetric procedures using kits #339, #352 and #352, respectively from Sigma Company. LDL combined with VLDL cholesterol were computed using the Friedwald algorithm (total cholesterol – HDL – (triacylglycerol/5)) (35). Inter assay cv (n=6) for plasma TAG and total cholesterol were 6.8% at 125 mg/dL and 4.6% at 200.0 mg/dL Homocysteine (HCy) concentrations from plasma were determined by RP-HPLC with fluorescence detection after pre-column derivatization with 7-fluoror-2,1,3-benzoxadiazole-4-sulfonate (36). Inter-assay coefficient of variations were 4–8% depending on the HCy concentration. Accuracy of this assay was assured by comparison to values determined in an expert laboratory (Dr. Pfeiffer, CDC). Our values in the concentration range 19–114  $\mu$ M deviated from those determined in the expert laboratory by 0.4% to 5.4%. Inter-assay cv ranged from 3.8% to 6.0% at 14.1–14.8  $\mu$ M while inter-assay cv ranged 0.6% to 4.1% at 9.2–31.0  $\mu$ M. 8-Hydroxydeoxyguanosine (8-OHdG) in lymphocytes was determined by RP-HPLC with electrochemical detection after DNA extraction from lymphocytes (37). Inter-assay cv for the ratio 8OHdG/10<sup>6</sup>dG was 6.2%. Detection limits were 0.6 nmol/L and 10  $\mu$ mol/L for 8OHdG and 10<sup>6</sup>dG, respectively.

Thiobarbituric acids reactive substances (TBARS) in plasma were analyzed spectrophotometrically and reported as malondialdehyde equivalents by reading the differences of the 535 nm and 572 nm absorbance of the products formed from plasma incubated with thiobarbituric acid reagent (38). This method avoids inclusion of false positive readings by excluding artifacts formed during plasma processing.

Laboratory personnel was blinded to the treatment status of the samples.

### Statistical analysis

Statistical evaluations regarding significance of changes from baseline were determined by paired t-test calculations using Microsoft Excel software (Office 2001, Microsoft Corporation, Redmond, WA) and by the Wilcoxon two sample rank sum tests using SAS software (SAS Institute, Inc., Cary, NC).

## RESULTS

The 4 batches of OJ used in this study were found to contain on average (mg/kg  $\pm$  standard deviation) 360  $\pm$ 8.1 ascorbic acid, 8.1  $\pm$ 0.16 carotenoids, 315  $\pm$ 25.3 hesperidin, 7  $\pm$ 0.7 hesperetin, 0.23  $\pm$ 0.02 folate, 54  $\pm$ 1.4 narirutin, and 6  $\pm$ 1.8 narirutin (table 1). At a dose of 3 cups (24 oz or 236.6 mL) per day this corresponded to a daily intake of 1452  $\mu$ moles of ascorbic acid, 12  $\mu$ moles of carotenoids, 0.37  $\mu$ moles folate, 383  $\mu$ moles hesperetin equivalents and 82  $\mu$ moles naringenin equivalents (citrus specific flavonoids). Among the carotenoids lutein and other xanthophylls predominated (66%) followed by cryptoxanthins (19%) and carotenes (15%). Approximately 90% of the xanthophylls were esterified compared to only 30% of the cryptoxanthins. Among the carotene isomers only the beta-isomer could be detected. In addition to beta-cryptoxanthin the alpha-isomer (21%) and cis-beta-isomer (13%) were identified.

Plasma after fasting for at least 8 hours was analyzed at baseline, after week 1 (day 8), and at the end of the intervention after week 3 (day 22). Significant changes were seen in some but not all of the individual carotenoids contained in OJ (figure 1, table 2). Mean *trans*-lutein and *trans*-zeaxanthin, reported together, significantly increased by 29% after week one from 271  $\mu$ g/L to 350  $\mu$ g/L and remained significantly elevated by 32% at week 3 (358  $\mu$ g/L). A similar trend was seen with *trans*-alpha-cryptoxanthin (33  $\mu$ g/L to 55  $\mu$ g/L to 70  $\mu$ g/L) and *trans*-beta-cryptoxanthin (124  $\mu$ g/L to 196  $\mu$ g/L to 240  $\mu$ g/L) equivalent to a 112% and 94% increase after the third week respectively. Also, concentrations of total *cis*-lutein plus *cis*-zeaxanthin, reported together, and *cis*-beta-cryptoxanthin significantly increased by week 3 from 125  $\mu$ g/

L to 143  $\mu\text{g/L}$  (a 15% increase) and from 36  $\mu\text{g/L}$  to 54  $\mu\text{g/L}$  (a 52% increase), respectively. Changes in the plasma concentrations of *trans*-anhydrolutein, *cis*-anhydrolutein, total lycopene, total dihydrolycopene and *cis*-beta carotene were not significant after 3 weeks (table 2). Alpha-carotene, and *trans*-beta-carotene increased significantly after week 1 but returned to baseline concentrations after week 3. Plasma concentrations of total carotenoids significantly increased by 20% from baseline (1282  $\mu\text{g/L}$ ) by week 1 (1537  $\mu\text{g/L}$ ) and remained significantly elevated by 22% at week 3 (1568 mg/ml) (figure 1). There was no significant change in the plasma concentrations of retinol or any of the tocopherols. Vitamin C and folate plasma concentrations were significantly higher after week 1 (74.8  $\mu\text{mol/L}$ =+62%; 55.7  $\mu\text{mol/L}$ =+44%), and after week 3 (73.1  $\mu\text{mol/L}$ =+59% ; 56.5  $\mu\text{mol/L}$ =+46%), than at baseline (46.1 $\mu\text{mol/L}$ ; 38.7  $\mu\text{mol/L}$ ), respectively (figure 2). Plasma concentrations of the flavonoids hesperetin and naringenin were significantly increased after week 3 by 512% and 874% respectively, from 3.3 nmol/L and 7.1 nmol/L, to 21.6 nmol/L and 68.7 nmol/L. The urinary excretion rates (measured in nmol per mg creatinine) of hesperetin and naringenin were also increased several fold and significantly increased by weeks 1 (21-fold and 4-fold) and week 3 (16-fold and 6-fold ) as compared with the baseline rates of 0.49 nmol/mg and 0.76nmol/mg, respectively. Urinary excretion of these analytes 2 days after the end of this study decreased back to baseline levels (data not shown). Mean homocysteine concentrations were elevated by 6% at week 3 (10.5  $\mu\text{mol/L}$ ,  $p=0.046$ ) as compared with the baseline (9.9  $\mu\text{mol/L}$ ) (figure 3). The mean 8-hydroxy deoxyguanosine:deoxyguanosine ratio, a marker of DNA damage was decreased, albeit insignificantly, and concentrations of thiobarbituric acid reactive substances, a marker of lipid oxidation, were almost unchanged (figure 3). Mean total cholesterol, HDL-cholesterol, and LDL-cholesterol concentrations were also little changed but there was a decrease in the mean LDL:HDL ratio by the end of the intervention (figure 4). This change, however, was not statistically significant. The mean TAG concentration on the other hand was significantly elevated by 26% at week 3 (1231 mg/L) as compared with the baseline (974 mg/L) (figure 4).

## DISCUSSION

In order to assess the effects of processing, handling methods, and storage conditions on the change of vitamin C, flavonoid, and carotenoid content in OJ (39-41), we monitored the concentration of these components in the OJ used during the trial. The micronutrient concentrations of the chilled not-from-concentrate OJ determined in our study are generally in agreement with those reported previously. There was minimal difference between the batches used. The vitamin C content (360 mg/kg) is close to that reported in the U.S. Department of Agriculture (USDA) food composition database ([www.nal.usda.gov/fnic/foodcomp](http://www.nal.usda.gov/fnic/foodcomp)). For example, chilled orange juices are listed to contain 330–427 mg/kg which is as expected lower due to processing related losses compared to the orange fruit (498 mg/kg ) or raw freshly squeezed juice (31). Carotenoid concentrations in raw juice were listed by USDA with almost identical concentrations compared to our findings. The inclusion of many polar carotenoids we included in the xanthophyll level caused most likely this value to be higher than the lutein/zeaxanthin concentration reported by USDA. Orange fruits were reported with the same pattern but with slightly lower absolute concentrations (41) which might be due to the non carotenoid containing compartments in the fruits which are removed in the juice and therefore concentrate carotenoids in the juice. In comparison with the total folate (290 mg/L) of chilled OJ listed in the USDA database, our value (231 mg/kg) was slightly lower. Flavonoids (naringenin, hesperetin) occur in OJ predominantly as glycosides (narirutin, hesperidin). The juice used in our study was found to contain 322 mg/kg hesperetin based and 60 mg/kg naringenin based flavanones. Less than 3% occurred as aglycones. These values are somewhat lower than those reported by Manach et al. (444 mg/kg and 96 mg/kg , respectively) (24) but higher than those found for 54 authentic orange juices (231 $\pm$ 42 mg/kg and 19 $\pm$ 121 mg/kg, respectively) (42) or the juice of 14 different orange cultivar juices (122–254 mg/kg and 18–56, respectively)

(43). This indicates that, as expected, variations in the content of the investigated phytochemicals in different orange juices exist.

Subjects' weight did not change during this study (data not shown). The participants reported lower intakes of snacks and smaller portion sizes of their meals due to consumption of the OJ spread over the entire day but assured that their diet in general did not change. The phytochemicals investigated and present in the OJ increased significantly in the circulation including carotenoids, vitamin C, folate, and the orange specific flavanons hesperetin and naringenin (fig. 1+2). While carotenoid and ascorbate concentrations in humans can be elevated by the intake of a variety of fruits and vegetables only specific vegetarian foods provide flavanones and the carotenoid cryptoxanthin. The elevation of circulating vitamin C concentrations by OJ intake has been reported repeatedly (21-23) including the claim that a citrus extract is superior compared to ascorbate supplements (44).

Our results showed a marked 22% and significant increase of circulating carotenoids after OJ intake which are in good agreement with other intervention studies using juices (45,46). Carotenoids are usually not well absorbed from the gut in the absence of lipids (10,47,48). The high carotenoid exposure during the entire day, the consumption of juices during meals that contained some fat, and the liquid nature of the exposure (presence of analytes in the dissolved state), situations that applied all or partly in our intervention, could explain the significant uptake of carotenoids into the circulation. In contrast to anemic schoolchildren who were found to have increased serum beta-carotene concentrations after orange fruit consumption (49) we did not observe a change in circulating alpha- or beta-carotene levels in our study. This might be due to the shorter intervention period (3 *versus* 9 weeks) and more importantly, to the differential nutritional status with the schoolchildren being malnourished while our study population was extremely well nourished maintaining a typical healthy Western diet before and during the study as estimated from interviews. Very few dietary items contain the carotenoid cryptoxanthin, for example oranges, mandarins, apricots, and papaya (41). The significant and very large increase (almost 100%) in plasma cryptoxanthins we observed in our intervention is particularly noteworthy considering the associations of this carotenoid in a case-control study with cervical cancer that showed a 20% and 70% decrease in risk between the highest *versus* the lowest plasma beta-cryptoxanthin and alpha-cryptoxanthin concentrations, respectively (50). In other, large epidemiologic studies dietary cryptoxanthin exposure was shown to be associated with reduced lung cancer risk of 15–31% (10,11). Similarly, protective effects were reported from Satsuma mandarins (*Citrus unshiu* Marc.), rich in beta-cryptoxanthin (and also hesperidin), which suppressed chemically-induced lung (51) and colon (52) carcinogenesis when fed to mice and rats, respectively.

Similar to the situation with cryptoxanthins few food plants produce significant amounts of flavanones typically occurring in citrus fruits (hesperetin and naringenin usually as glycosides). We found a several fold increase of plasma levels and urinary excretion of hesperetin during the intervention which returned to baseline two days after study completion. This is in excellent agreement with the fast pharmacokinetics of these flavonoids (24,34,53-55). We observed that more naringenin compared to hesperetin related flavanones were retained in the circulation. This is similar to our previous findings on the soy isoflavones genistein which is retained more in the circulation and excreted at a lower rate than daidzein due to its lower polarity (56). The health benefits of flavonoids have been intensively investigated (reviewed in (57)) and the citrus flavanons are no exception (58). We reported earlier on the potent effect of citrus flavonoids to inhibit neoplastic transformation (59) and also on their preventive effects on formation of colonic aberrant crypt foci, a surrogate marker for colon cancer, in rats (60). In addition to protecting against cancer, preventive effects for heart disease seem likely (61) but conclusive results from epidemiologic and clinical studies are so far not available. Health

benefits of these flavonoids are probably potentiated by combinations with other phytochemicals occurring in plant foods, particularly carotenoids (51,52).

DNA damage in white blood cells decreased in our study by 16% and in individuals with high baseline concentrations by 29% (n=7) however, this did not reach statistical significance. The marker of lipid oxidation (TBARS) changed minimally. Intervention studies with single or multiple supplementations of vitamin C, vitamin E, or carotenoids and those involving various natural food products were found to yield mixed results (reviewed in (62)). In short-term intervention studies (usually weeks or a few months), single-dosing studies found that decreased oxidative (DNA) damage lasted only hours after antioxidant supplementation. Since we collected plasma after subjects had been fasting for at least 8 hours we might have missed the optimal time when the antioxidant effect was measurable.

In previous studies daily consumption of 8 fl. oz. OJ for a 2-week period or supplemental vitamin C (approximately 70 mg/day) effectively raised circulating vitamin C and reduced TBARS (23). But the investigated cohort had a very low vitamin C status (22–28  $\mu\text{mol/L}$ ) and high TBARS readings at baseline rendering them susceptible to the expected changes. Commercial fresh-squeezed OJ (500 mL/day) given to 12 subjects for 14 days increased plasma concentrations of vitamin C and reduced concentrations of 8-epi-PGF(2)-alpha, another marker for lipid oxidation (22). We did not measure this isoprostane in our study, and it is conceivable that this marker reflects more selectively and sensitively changes in the systemic antioxidant status. More importantly, we believe that our study population had a high antioxidant status at baseline due to the high baseline levels of antioxidant micronutrients in plasma (see tables and graphs), and their relatively low baseline levels of markers of oxidative damage. Therefore, an increase in antioxidants in these subjects may lead to insignificant changes in markers of oxidative damage. Previously, we determined repeatedly in nine individuals over a 3-month period levels of TBARS and found positive correlations with plasma triglycerides and gamma-tocopherol, and negative correlations with plasma carotenoids (63). The significant TAG increase in this study (26%) may have led to higher TBARS and thereby offset the TBARS lowering effect of the antioxidants. The increase in plasma TAG concentrations (26%) may be due to the increase in total carbohydrate intake (64). The elevation did not pose a risk though because it was well below critical values. A very similar increase in plasma TAG concentrations (30%) was reported from an intervention trial that used the same, not-from concentrate OJ at almost the same daily dose for 4-weeks with 25 hypercholesteremic subjects (21). In contrast to that study, we found that mean total as well as HDL and LDL cholesterol concentrations did not change markedly while the LDL:HDL ratio did favorably. However, no statistical significance was reached for any of these changes. These discrepant results are probably due to the different study population. OJ might be more effective in decreasing cholesterol levels in hypercholesteremic but not in normocholesteremic subjects. This is similar to the effect seen with soy protein exposure (65) and seems to be a desirable effect because lowering normal levels could lead to critical ranges with potentially adverse outcomes.

Despite elevated folate concentrations (46% *versus* baseline) homocysteine (HCy) concentrations in the circulation did not decrease in our study. We found surprisingly that total HCy plasma concentrations increased (6%,  $p=0.05$ ) which is in contrast to the traditional belief that folate exposure will lower HCy concentrations (66). However, HCy was found to be significantly higher in vegetarian subjects than in controls independent of folate status (67). Also, C667T transitions in the methylenetetrahydrofolate reductase (MTHFR) gene affect diet responsiveness of plasma HCy upon folate exposure (68). About half of the interindividual HCy variability in response to folic acid could be explained by basal HCy, age, male gender, cigarette smoking, use of multivitamins, MTHFR or cystathionine beta-synthase polymorphisms, but the other half could not be explained (69), and individual variability in HCy response to folate depletion is much investigated (70). Our finding might therefore be

due to cofounders including the possible change of the participants to a more plant based diet during the intervention, the vast interindividual variation of HCy change upon folate exposure, or a paradoxical response to folate intake (69). Our study did not allow stratification for these variables. In addition, the HCy levels in our group were relatively low at baseline and therefore difficult to reduce further. Furthermore, the high antioxidant status of our study population at baseline, a possible saturation effect due to folic acid food fortification that took effect shortly before this study started, the relatively short period of intervention, or other unknown factors might also have contributed to our unusual finding. The present study shows a marked increase in dietary antioxidants and a decrease in some but not all measured risk markers. Due to the limitations of this study are the relatively low numbers of participants, the relatively short period of intervention, and the non-controlled diet. Although our interviews indicated no significant changes in the diet during the intervention the present results of this study need to be confirmed in larger trials with higher statistical power which could lead to more precise conclusions regarding the effects of OJ on prevention of oxidative damage. The lack of reduction in markers of oxidative damage in this study might be due to the high antioxidant status of the participants at baseline, to changes in phytochemicals that do not affect the measured markers, or are minor in comparison to the massive concentrations of other systemic antioxidants (proteins, bilirubin, urate, etc.) (71). Despite our observation on increased HCy and TAG levels, our results in combination with data from other OJ interventions suggest strongly that OJ is an excellent food source to increase blood levels of a series of valuable phytochemicals. These can then exert their activity by various mechanisms including a decrease in oxidative damage to select biological targets and thereby protect against chronic disorders especially, in at-risk populations with low antioxidant status. The increase in circulating cryptoxanthins we observed for the first time after OJ exposure is particularly intriguing because concentrations almost doubled in this study after delivery from juice which is traditionally believed to be a route that leads to poor bioavailability of lipid phase micronutrients. Therefore, we conclude that OJ is an excellent source to provide carotenoids and flavonoids which occur in few fruits and vegetables at the same high levels and composition and which may protect against cancer and other chronic diseases.

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## Biography

A. Franke had the overall lead of this study which included to design the protocol, recruit volunteers, design the consent form, provide for IRB approval, obtain written consent of volunteers, perform the intervention, evaluate the data, and prepare the manuscript. with graphs and figures.. Cooney performed the TBARS assay and consulted in antioxidant related matters. S. Henning performed the 8OHdG assay and consulted in antioxidant related matters. L. Custer processed collected blood and urine specimen and performed all lab assays except those mentioned above and for TBARS, 8OHdG, and folate.

## ABBREVIATIONS USED

8OHdG, 8-Hydroxy deoxyguanosine  
a-CAR, trans alpha-carotene  
a-CRX, trans-alpha-cryptoxanthin



b-+g TOC, beta and gamma tocopherol  
 b-CAR, trans beta-carotene  
 cis LUT/ZEA, total (cis-lutein+cis-zeaxanthin)  
 cis AH-LUT, cis-anhydrolutein  
 cis b-CAR, cis beta-carotene  
 cis b-CRX, cis alpha-cryptoxanthin  
 CHOL, cholesterol  
 CRCH, Cancer Research Center of Hawaii  
 cv, coefficient(s) of variation  
 dG, deoxyguanosine  
 d-TOC, delta tocopherol  
 DH-LYC, total dihydrolycopene  
 HCy, homocysteine  
 HDL, high density lipoprotein  
 HPLC, high pressure liquid chromatography  
 LC, liquid chromatography  
 LC/MS, liquid chromatography mass spectrometry  
 LDL, low density lipoprotein  
 LYC, total lycopene  
 MTHFR, methylenetetrahydrofolate reductase  
 NIST, National Institute for Standards and Technologies  
 OJ, orange juice  
 PDA, photo diode array detection  
 TBARS, thiobarbituric acid reactive substances  
 TAG, triacylglycerols  
 tr-LUT/ZEA, trans-lutein+trans-zeaxanthin  
 tr-AH-LUT, trans-anhydrolutein  
 tr b-CRX, trans beta- cryptoxanthin  
 USDA, U.S. Department of Agriculture  
 UV/VIS, ultra-violet/visible

## References

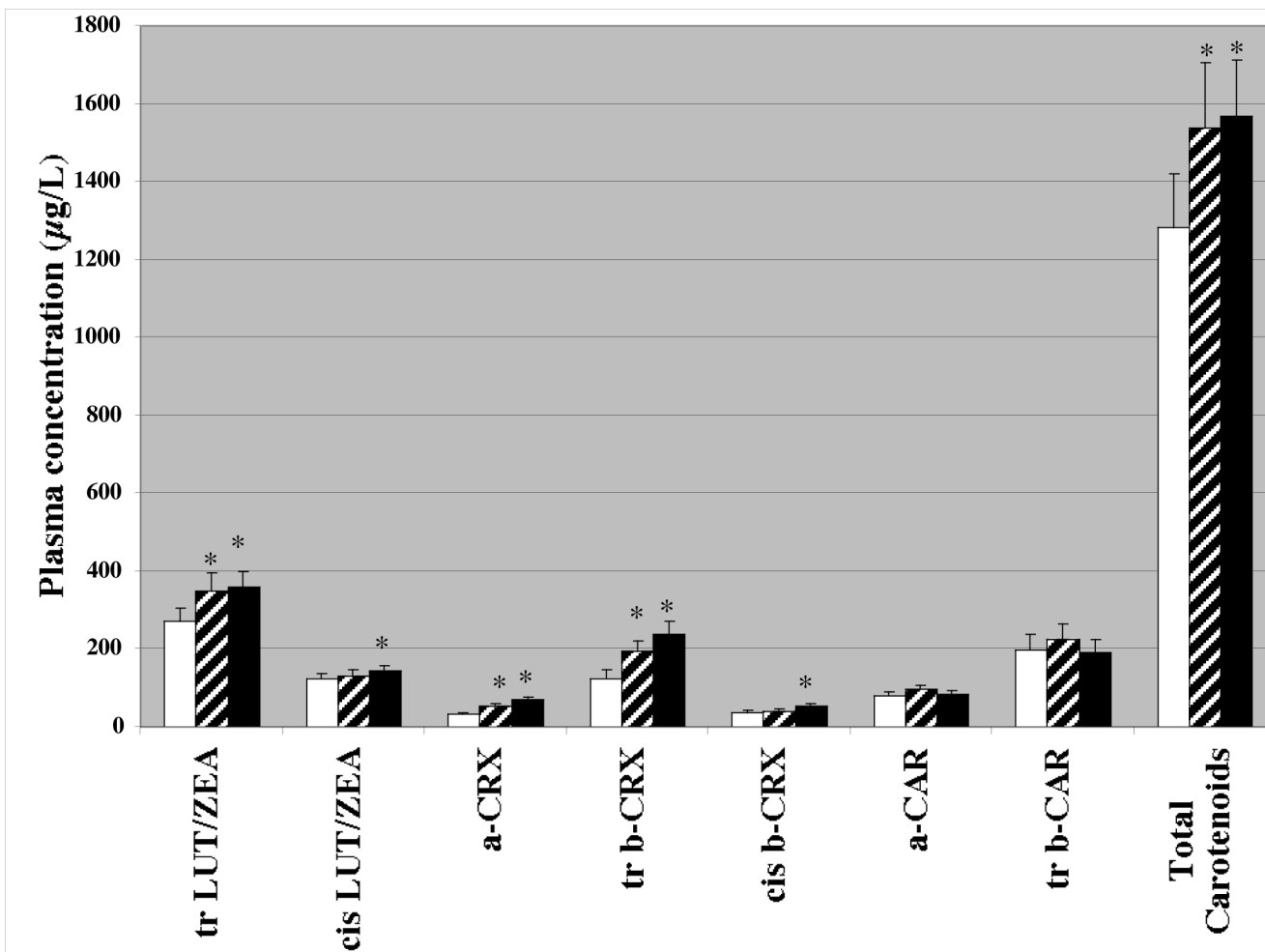
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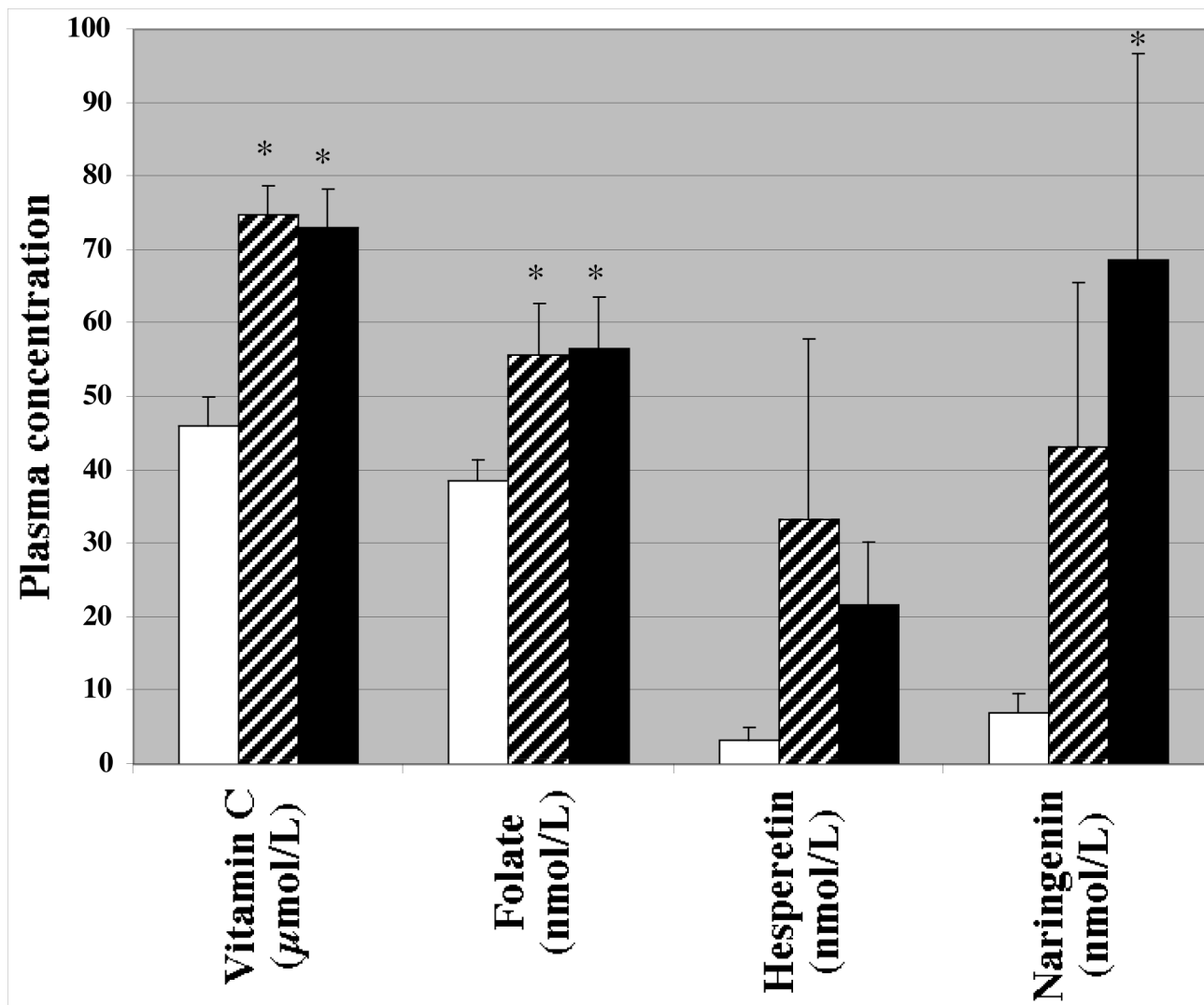
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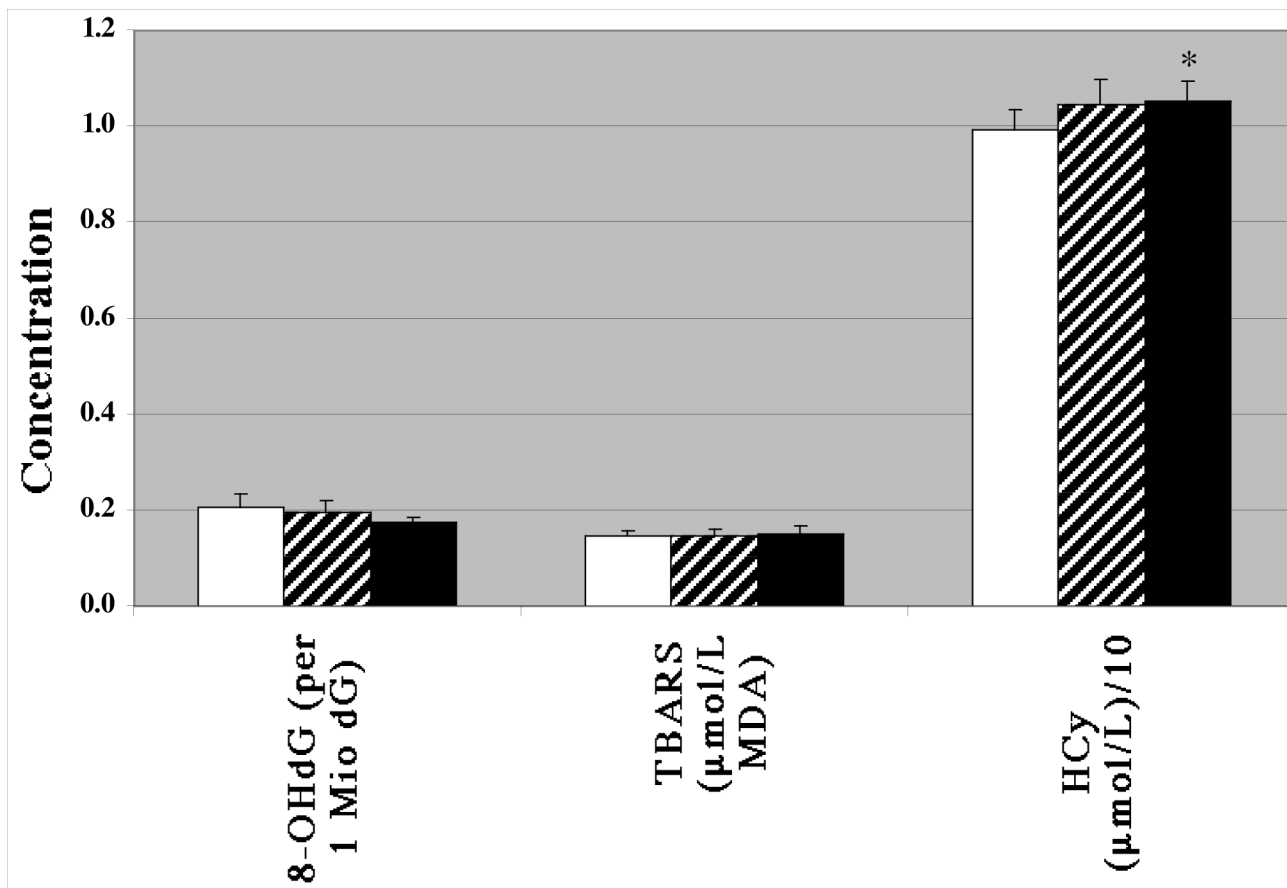
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**Figure 1.** Mean plasma concentration change of individual and total carotenoids. t-LUT/ZEA=trans-lutein+trans-zeaxanthin; cis-LUT/ZEA=total cis-lutein+cis-zeaxanthin; a-CRX= alpha-cryptoxanthin; tr beta-CRX=trans beta-cryptoxanthin; cis beta-CRX=cis beta-cryptoxanthin; a-CAR= alpha-carotene; tr beta-CAR=trans beta-carotene. White bars = baseline, striped bars = after 1 week, black bars = after 3 weeks. Error bars indicate standard error. To convert into µmol/L see legend of table 2. \* p<0.05 for paired t-test comparing to baseline.

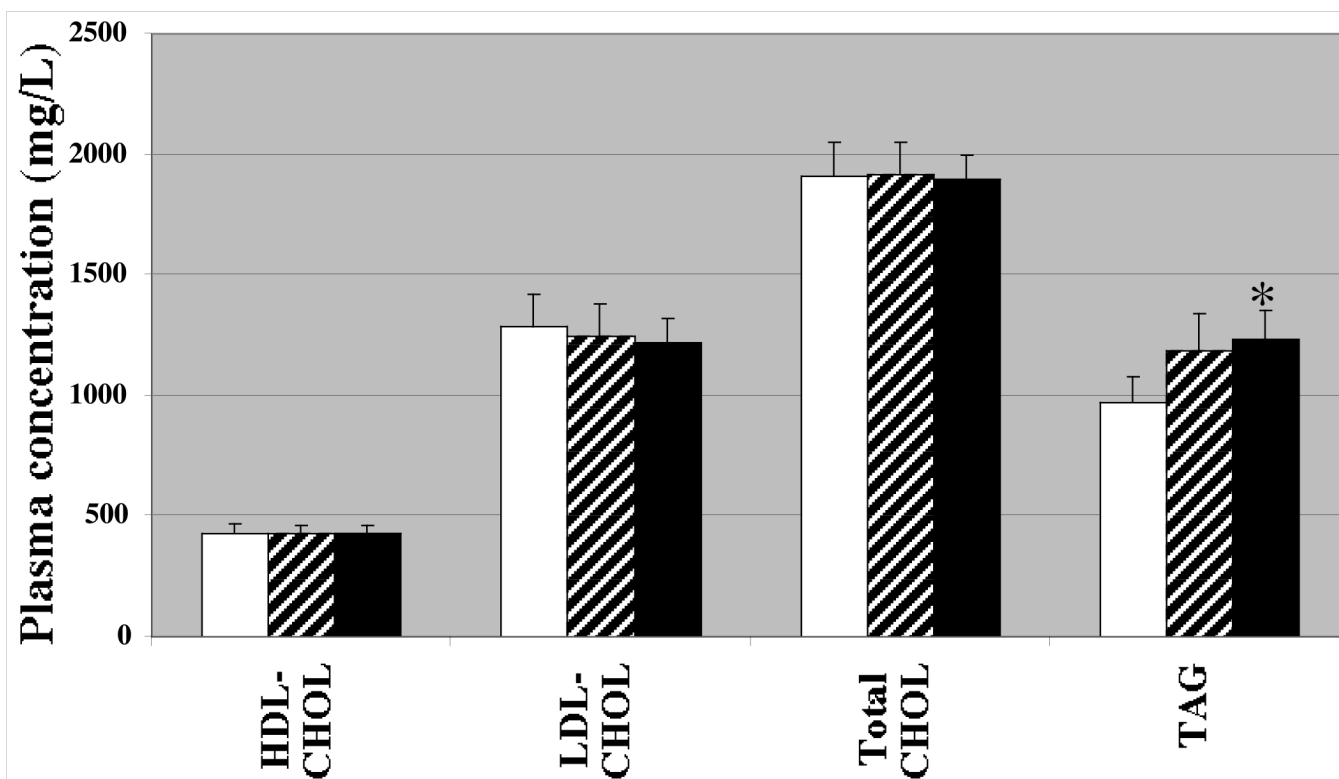


**Figure 2.** Mean change in plasma concentrations of ascorbic acid, folic acid, and the flavonoids hesperetin and naringenin. Glucuronide and sulfate conjugates of flavonoids occurring in plasma were enzymatically hydrolyzed prior to extraction and LC/MS analysis. White bars = baseline, striped bars= after 1 week, black bars = after 3 weeks. Error bars indicate standard error. \*  $p < 0.05$  for paired t-test comparing to baseline.



**Figure 3.** Mean change of the 8-hydroxy deoxyguanosine (8 -OHdG)/deoxyguanosine (dG) ratio in white blood cells and change in plasma concentrations of thiobarbituric acid reactive substances (TBARS) and homocysteine (HCy). The latter values are 10 times greater than displayed in graph. White bars = baseline, striped bars= after 1 week, black bars = after 3 weeks. Error bars indicate standard error. TBARS units are malondialdehyde (MDA) equivalents. HCy values were divided by 10 to fit scale of graph. \* p<0.05 for paired t-test comparing to baseline.





**Figure 4.** Mean plasma concentration change of total, HDL and LDL cholesterol, and of triacylglycerols (TAG). White bars = baseline, striped bars= after 1 week, black bars = after 3 weeks. Error bars indicate standard error. To convert into  $\mu\text{mol/L}$  see legend of table 2. \*  $p < 0.05$  for paired t-test comparing to baseline.

**Table 1**

Composition, dose, and variability of the not-from-concentrate OJ used in this study.

	<u>juice concentration</u> <u>mg/kg</u>	<u>Dose</u> <u>mg/d</u>	<u>μmol/d</u>	<u>CV</u> <sup>*</sup> <u>%</u>
Vitamin C	360	255.5	1451.9	2.3%
Carotenoids	8.10	5.7	11.5	18.5%
Xanthophylls <sup>1</sup>	5.40	3.8	6.7	18.1%
Cryptoxanthins <sup>2</sup>	1.59	1.1	2.0	16.2%
Trans beta-carotene	0.40	0.3	0.5	22.2%
Hesperidin+Hesperetin	322	228.6	382.7	7.1%
Narirutin	60	42.6	81.7	2.5%
Folate	0.23	0.16	0.37	9.2%

\* coefficient of variation between 2 different batches of juice and 4 different samples

<sup>1</sup> mainly lutein and zeaxanthin

<sup>2</sup> 75% trans beta-cryptoxanthin, 6% cis beta-cryptoxanthin, 19% alpha-cryptoxanthin

**Table 2**  
Baseline concentrations and mean changes in the circulation during this study

	level at baseline	change versus baseline	
		1 week (p-value <sup>*</sup> )	3 weeks (p-value <sup>*</sup> )
tr LUT/ZEA (µg/L)	270.5	<b>29% (0.041)</b>	<b>32% (0.001)</b>
cis LUT/ZEA (µg/L)	124.6	5% (0.359)	15% ( <b>0.034</b> )
tr AH-LUT (µg/L)	64.3	-3% (0.336)	-1% (0.741)
cis AH-LUT (µg/L)	44.3	-4% (0.443)	2% (0.675)
a-CRX (µg/L)	33.1	<b>67% (&lt;0.001)</b>	<b>112% (&lt;0.001)</b>
tr b-CRX (µg/L)	123.7	<b>58% (&lt;0.001)</b>	<b>94% (&lt;0.001)</b>
cis b-CRX (µg/L)	35.8	12% (0.059)	<b>52% (&lt;0.001)</b>
LYC (µg/L)	230.0	<b>-9% (0.027)</b>	5% (0.624)
DH-LYC (µg/L)	64.5	-14% (0.015)	-3% (0.715)
a-CAR (µg/L)	79.1	21% (0.034)	8% (0.387)
tr b-CAR (µg/L)	198.9	14% (0.039)	-4% (0.549)
cis b-CAR (µg/L)	13.1	-3% (0.555)	-3% (0.612)
Total Carotenoids (µg/L)	1281.9	<b>20% (0.004)</b>	<b>22% (&lt;0.001)</b>
Retinol (µg/L)	746.4	15% (0.121)	16% (0.051)
d-TOC (µg/L)	405.1	-3% (0.051)	-20% (0.051)
b+g-TOC (µg/L)	1566.3	-5% (0.523)	-6% (0.558)
a-TOC (µg/L)	8159.0	0% (0.979)	4% (0.349)
Ascorbic acid (µmol/L)	46.7	<b>62% (&lt;0.001)</b>	<b>59% (&lt;0.001)</b>
Folic acid (nmol/L)	38.7	<b>44% (0.013)</b>	<b>46% (0.018)</b>
Hesperetin (nmol/L)	3.3	1016% (0.227)	<b>657% (0.045)</b>
Naringenin (nmol/L)	7.1	612% (0.113)	<b>974% (0.044)</b>
8OHdG/10 <sup>6</sup> dG	0.2	-5% (0.755)	-16% (0.204)
TBARS (µmol/L MDA)	0.2	0% (0.970)	2% (0.795)
HCy (µmol/L)	9.9	5% (0.124)	<b>6% (0.046)</b>
HDL-CHOL (mg/L)	431.4	-1% (0.814)	-1% (0.743)
LDL-CHOL (mg/L)	1286.7	-3% (0.365)	-5% (0.350)
Total CHOL (mg/L)	1912.9	0% (0.986)	-1% (0.827)
TAG (mg/L)	974.0	22% (0.147)	<b>26% (0.016)</b>
LDL/HDL	3.3	-3% (0.474)	-3% (0.498)

t-LUT/ZEA=trans-lutein+trans-zeaxanthin (µg/L), cis-LUT/ZEA=total (cis-lutein+cis-zeaxanthin,t-AH-LUT=trans-anhydrolutein, cis-AH-LUT=cis-anhydrolutein, a-CRX=trans-alpha- cryptoxanthin, tr b-CRX=trans beta-cryptoxanthin, cis b-CRX=cis alpha-cryptoxanthin, LYC=total lycopene, DH-LYC=total dihydrolycopene, a-CAR=trans alpha-carotene, b-CAR=trans beta-carotene, cis b-CAR=cis beta-carotene, d-TOC=delta tocopherol, b-+g TOC=beta and gamma tocopherol, 8OHdG=8-hydroxydeoxyguanosin, TBARS=thiobarbituric acid reactive substances. HCy=homocysteine, CHOL=cholesterol, TAG=triacylglycerols; HDL=high density lipoprotein, LDL=low density lipoprotein. To convert into µmol/L multiply µg/L by 0.00176 (LUT/ZEA), 0.00181 (CRX), 0.00187 (CAR, LYC), 0.00349 (Retinol), 0.00232 (aTOC), 0.00240 (b+gTOC), 0.00248 (dTOC) or multiply mg/L by 2.59 (CHOL) and 1.13 (TAG), or divide nmol/L by 1000.

\* p-value determined by student's paired t-test, **bold indicates p<0.05.**