Food Chemistry 173 (2015) 1187–1194

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

The intake of broccoli sprouts modulates the inflammatory and vascular prostanoids but not the oxidative stress-related isoprostanes in healthy humans

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ARTICLE INFO

Article history:
Received 10 September 2013
Received in revised form 29 October 2014
Accepted 31 October 2014
Available online 7 November 2014

Keywords:
Sulforaphane
Vitamin C
Urine
Eicosanoids
Oxidative stress
Broccoli

ABSTRACT

Current evidence supports the positive association between the consumption of plant foods and health. In this work, we assessed the effect of consuming a half-serving (30 g) or one serving (60 g) of broccoli sprouts on the urinary concentrations of biomarkers of oxidative stress (isoprostanes) and inflammation (prostaglandins and thromboxanes). Twenty-four volunteers participated in the project. A quantitative determination of sulforaphane and its mercapturic derivatives, eicosanoids, and total vitamin C in urine was performed. The intake of broccoli sprouts produced an increase in the urinary concentrations of sulforaphane metabolites and vitamin C. Among the 13 eicosanoids analyzed, tetranor-PGEM and 11β-$PGF_{2\alpha}$ as well as 11-dehydro-TXB_2 showed a significant decrease in their urinary concentrations after the ingestion of broccoli sprouts. Therefore, the consumption of broccoli sprouts modulated the excretion of biomarkers linked to inflammation and vascular reactions without exerting a significant influence on the oxidation of phospholipids *in vivo*.

1. Introduction

The eicosanoids constitute a group of oxygenated products of long-chain polyunsaturated fatty acids, formed enzymatically or non-enzymatically through the oxidation of arachidonic acid and esterification of cell membrane lipids (Morrow et al., 1990, 1994). This family includes isoprostanes (IsoPs), leukotrienes (LTs), prostaglandins (PGs), and thromboxanes (TXs), which act as lipid mediators involved in the pathophysiology of distinct organs, tissues, and cells (Medina, Domínguez-Perles, Cejuela-Anta, et al., 2012). These lipid mediators are mainly involved in oxidative stress (IsoPs) and in homeostatic biological functions and inflammation (prostanoids (PGs and TXs)) (Morrow et al., 1990, 1994; Pérez-Sala, 2011). After their synthesis, IsoPs and prostanoids are esterified and/or bioconverted into free acid forms and spread throughout the organism (Kaviarasan, Muniandy, Qvist, & Ismail, 2009), being excreted in urine (Morrow et al., 1999).

Thus, the IsoPs have been highlighted as informative biochemical variables linked to oxidative reactions and their biological synthesis and excretion undergo changes in response to a number of pathophysiological processes including age-related diseases, cardiovascular disease, cancer, neurological disorders, and others (Cracowski et al., 2000; Roberts Ii & Morrow, 1997). Moreover, prostanoids (PGs and TXs) are involved in homeostatic mechanisms closely linked to inflammation, fever, and pain (Blatnik & Steenwyk, 2010). The possibility of determining their urinary concentrations turns them into potential valuable, non-invasive markers to assess variation of *in vivo* oxidative and inflammatory phenomena and, therefore, useful tools for the assessment of pathophysiological states in humans. Likewise, the modification of their concentrations in distinct organs may be indicative of biological

Abbreviations: AA, ascorbic acid; BIS–TRIS, bis-(2-hydroxyethyl)-amino–tris(hydroxymethyl)-methane; COX, cyclooxygenase; CV, coefficient of variation; DHAA, dehydroascorbic acid; ESI, electrospray ionization; FDA, Food and Drug Administration; Fw, fresh weight; GR, glucoraphanin; HPNE, 4-hydroperoxy-2-nonenal; ICH, International Conference on Harmonization; IsoP, isoprostane; MRM, Multiple Reaction Monitoring; LC–MS, liquid chromatography–mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; OPDA, 1,2-orthophenylendiamine; PG, prostaglandin; SFN, sulforaphane; SFN-Cys, sulforaphane cysteine; SFN-GSH, sulforaphane glutathione; SFN-NAC, sulforaphane *N*-acetylcysteine; TX, thromboxane; UHPLC–QqQ–MS/MS, Ultra high pressure liquid chromatography–triple quadrupole–tandem mass spectrometry.

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http://dx.doi.org/10.1016/j.foodchem.2014.10.152
0308-8146/

activity upon external interventions – including those performed during dietary programs (Comporti et al., 2008; Milatovic, Montine, & Aschner, 2011).

Brassica foods are of particular note due to their content of glucosinolates, which are cleaved enzymatically to form their cognate bioactive isothiocyanates. Broccoli sprouts (*Brassica oleracea* L. var. *Italica*) are the main dietary source of glucoraphanin (GR), the sulforaphane (SFN), glucosinolate (Pérez-Balibrea, Moreno, & García-Viguera, 2008) produced by the enzymatic cleavage catalyzed by myrosinase (β-glucuronidase glucohydrolase), an enzyme present in the same plant material, or – less effectively – by the microbial glucosidases (Clarke et al., 2011a). Besides glucosinolates, broccoli sprouts are rich in phenolic acids, vitamins (A, C, E, and K), and minerals, making this food an interesting source of healthy compounds (West et al., 2004). Previous work supported the anti-cancer properties of SFN, by *in vitro* and *in vivo* studies with humans and animals (Clarke, Dashwood, & Ho, 2008; Dinkova-Kostova, & Kostov, 2012). Additionally, natural antioxidants such as vitamin C, also found in broccoli sprouts, have been related to the reduction of inflammation and oxidative stress (Holt et al., 2009). However, the ability of these bioactive compounds to modulate oxidative stress, inflammation, and vascular pathophysiology remains poorly studied in humans.

The aim of this work was to investigate the effects of the consumption of half (30 g) and full (60 g) servings of fresh broccoli sprouts on biomarkers of oxidative stress, inflammation, and vascular pathophysiology, as well as the relationship of their urinary concentrations with the bioavailability of the mercapturic conjugates of SFN and vitamin C.

2. Materials and methods

2.1. Reagents

All LC–MS grade solvents were obtained from J.T. Baker (Phillipsburg, New Jersey, USA) and BIS–TRIS (bis-(2-hydroxyethyl)-amino–tris(hydroxymethyl)-methane) was purchased from Sigma–Aldrich (St. Louis, Missouri, USA). Formic acid was purchased from Panreac (Castellar Del Vallés, Barcelona, Spain). The Strata solid phase extraction (SPE) cartridges (Strata X and X-AW, 100 mg, 3 mL^{-1}) were from Phenomenex (Torrance, California, USA). C_{18} Sep-Pak cartridge used in the SPE previous to the analysis of vitamin C was purchased from Waters (Milford, MA, USA). The GR and SFN were purchased from CRA-CIN (Rome, Italy) and Sigma (St. Louis, MO, USA), respectively. The standards of SFN-glutathione, SFN-cysteine, and SFN-*N*-acetylcysteine (SFN-GSH, SFN-Cys, and SFN-NAC, respectively) were from SantaCruz Biotech (CA, USA). Ascorbic acid (AA) and dehydroascorbic acid (DHAA) were purchased from Sigma (St. Louis, MO, USA) and 1,2-orthophenylendiamine (OPDA) was purchased from Fluka Chemika (Neu-Ulm, Switzerland), respectively. Five isoprostanes (The 8-iso $PGF_{2\alpha}$; 8-iso-15(R)-$PGF_{2\alpha}$; 2,3-dinor-8-iso-$PGF_{2\alpha}$; 2,3-dinor-11β-$PGF_{2\alpha}$; and 8-iso-15-keto $PGF_{2\alpha}$), seven prostaglandins (11β-$PGF_{2\alpha}$; 9,11-dideoxy-9α,11α-methanoepoxy $PGF_{2\alpha}$ (U-46619); 9,11-dideoxy-9α,11α-epoxymethano $PGF_{2\alpha}$ (U-44069); 2,3-dinor-6-keto $PGF_{1\alpha}$ (sodium salt); 6-keto $PGF_{1\alpha}$; tetranor-PGFM (tetranor-PGF-metabolite), and tetranor-PGEM (tetranor-PGE-metabolite)), and one thromboxane (11-dehydro thromboxane B_2) were from Cayman Chemicals (Ann Arbor, Michigan, USA). The β-glucuronidase, type H2 from *Helix pomatia*, was provided by Sigma–Aldrich (St. Louis, Missouri, USA).

2.2. Physical characteristics of participants and composition of broccoli sprouts

The protocol was approved by the Bioethics Committee of the University Hospital of Murcia and all participants provided written, informed consent to the Institution (Speid, 2010). Twenty-four Caucasian volunteers (12 women and 12 men) agreed to participate in the project. The subjects maintained their usual lifestyles during the study. The participants were non-smokers, followed normalized standard diets (the volunteers filled out a food questionnaire) and did not receive any medication during the experimental procedure. The volunteers were subjected to a pharmacology test (both prescription and over-the counter medication). Participants were apparently healthy and had a normal medical history and physical examination. During the study, the women were not in menstrual days (menstrual bleeding), a factor that could increase the urinary eicosanoids concentration. The physical parameters and dietary consumption of the volunteers were strictly controlled and are listed in Table 1. The nutritional composition and energy value of the dietary intake, including broccoli sprouts intake (1/2 and 1 serving) and the control group, are summarized in Table 1 (data calculated by the software available on the website http://www.invesalia.es/evaluacion/), with the additional assistance of the Spanish and USDA databases (http://www.bedca.net/) and http://www.nal.usda.gov/fnic/foodcomp/search/). The nutritional intake and the additional nutrients and phytochemicals provided by the broccoli sprouts were accurately determined to establish the normalized diet for all the volunteers. All participants ($n = 24$) in this study avoided consumption of Brassica foods in the week previous to the onset of the nutritional assay; this was used as the wash-out period. The clinical study lasted three days and 12 volunteers from the 24 people involved followed the same diet, free of broccoli sprouts, during this period. In parallel, the other 12 volunteers followed a 3-day crossover nutritional study (1 day intake + 1 day wash out + 1 day intake). According to this scheme and during the days of intake, six volunteers consumed one serving of raw, fresh broccoli sprouts (60 g) and six ingested a half-serving (30 g), as shown in Fig. 1 and Table 1.

Raw, fresh broccoli sprouts, produced organically following the method of Pérez-Balibrea et al. (2008), were donated by Aquaporins & Ingredients S.L. (Murcia, Spain). The volunteers ingested 30 or 60 g of fresh broccoli sprouts during different periods. These amounts are consistent with a half- and one serving, respectively (Food and Drug Administration (FDA), 2001).

Freeze-dried samples of broccoli sprouts (100 mg) were extracted, following previously-described methodology for the determination of their intact GR (Pérez-Balibrea et al., 2008) and SFN (Table 2).

In order to determine the relative variation in the concentrations of the target analytes in control urines, in comparison with the urines of volunteers that consumed a half- or one serving of broccoli sprouts, the control urines were collected time-matched with volunteers who ingested broccoli sprouts (0–12 h and 12–24 h) (Fig. 1). The urines were collected and frozen at −80 °C for further analysis. The dietary intervention was at 10:00, with no meal in the 2 h before and after the intervention. Urine was collected from 10:00 to 22:00 (0–12 h) and from 22:00 to 10:00 of the following day (12–24 h), to avoid the influence of the circadian system on the metabolic settings. The total urine volume was recorded, to calculate the absolute amounts of the analytes excreted in the study period.

2.3. Urine samples

To determine the total content of SFN, IsoPs, and prostanoids, the samples were thawed at room temperature and centrifuged (11,000×*g*, 5 min). To assess the concentrations of SFN and its mercapturic derivatives, urine samples (1 mL) were extracted by SPE using Strata-X cartridges (33u Polymeric Strong Cation; Phenomenex, CA, USA) – following the procedure described in the

Table 1
Characteristics of volunteers involved in the study and dietary parameters during the intervention period.

Physical parameters	Control volunteers (n = 12)	Volunteers consuming ½ serving (n = 6)	Volunteers consuming 1 serving (n = 6)
Age	35.8 ± 11.5	34.0 ± 10.0	34.0 ± 6.0
Height (m)	1.70 ± 0.08	1.69 ± 0.06	1.74 ± 0.08
Weight (kg)	69.8 ± 18.7	70.7 ± 12.3	73.3 ± 20.4
BMI (kg m^{-2})[Y]	24.1 ± 3.1	24.7 ± 3.3	23.9 ± 5.3
Dietary parameters	Control diet nutritional composition	Broccoli sprouts (1 serving) nutritional composition[X]	Broccoli sprouts (½ serving) nutritional composition[X]
Energy intake (kcal d^{1})	2000.0 ± 196.3	2156.3 ± 233.7 (∼7.2%)	2078.1 ± 215.1 (∼3.7%)
Carbohydrate (g d^{-1})	260.0 ± 21.6	262.1 ± 21.6 (∼0.8%)	261.05 ± 19.8 (∼0.4%)
Dietary fiber (g d^{-1})	25.0 ± 0.7	25.6 ± 0.7 (∼2.3%)	25.3 ± 0.6 (∼1.2%)
Proteins (g d^{-1})	50.0 ± 1.3	51.0 ± 1.4 (∼2.0%)	50.5 ± 1.3 (∼1.0%)
Total lipids (g d^{-1})	70.0 ± 4.7	70.8 ± 4.8 (∼1.1%)	70.4 ± 4.7 (∼0.5%)
Vitamin C (mg d^{-1})	60.0 ± 4.2	98.9 ± 5.5 (∼39.3%)	79.5 ± 4.8 (∼24.4%)
Vitamin E (mg d^{-1})	15.0 ± 0.02	15.7 ± 0.12 (∼4.5%)	15.3 ± 0.11 (∼2.3%)
Selenium (μg d^{-1})	50.0 ± 0.9	52.4 ± 1.5 (∼4.6%)	51.1 ± 0.9 (∼2.1%)
Zinc (μg d^{-1})	10.0 ± 0.05	170.4 ± 17.5 (∼94.1%)	90.2 ± 8.7 (∼88.9%)
Glucoraphanin (GR) (mg)	–	101.9 ± 7.5 (∼100%)	51.3 ± 3.0 (∼100%)
Sulforaphane (SFN) (mg)	–	7.4 ± 0.6 (∼100%)	3.5 ± 0.2 (∼100%)

Data are represented by mean ± SD.
The volumes of excreted urine corresponded to the control group (0–12-h/12–24-h) and those volunteers that ingested ½ serving of BS (0–12-h/12–24-h) and 1 serving of BS (0–12-h/12–24-h) were 1140/255 and 1247/667 and 967/553, respectively.
[X] The percentages of the nutritional values provided by the broccoli sprouts respected to the control diet are indicated between brackets.
[Y] Body mass index.

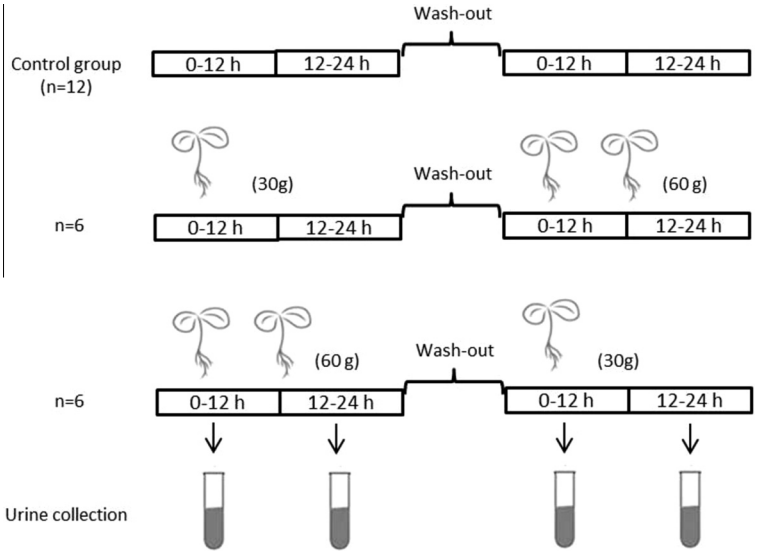

Fig. 1. Assay design of the nutritional study.

UHPLC–QqQ–MS/MS section. Regarding IsoPs and prostanoids, the urine samples were first hydrolyzed and extracted according to the procedure described by Medina, Domínguez-Perles, Cejuela-Anta, et al., (2012), Medina, Domínguez-Perles, Gil, et al. (2012).

2.4. UHPLC–QqQ–MS/MS analyses of the isothiocyanate metabolites. Validation of the method

The urine samples (400 μL) for determination of the free isothiocyanates and the solvents were spiked with the stock solution of GR, SFN, and the mercapturic acid derivatives of SFN, to achieve a concentration of 2500 nmol L^{-1} for each compound. The standards were extracted using SPE Strata-X cartridges (33u Polymeric Strong Cation) following the manufacturer's instructions (Phenomenex, Torrance, CA, USA). Briefly, the cartridges were conditioned with 2 mL of MeOH and equilibrated with 2 mL of ultrapure water/formic acid (98:2, *v/v*). After this step, the urine samples were diluted in 2 mL of water/formic acid (98:2, *v/v*) and applied to the column. Then, the SPE cartridges were washed with water/formic acid (98:2, *v/v*) and aspirated until dryness. The target analytes were eluted with 1 mL of MeOH/formic acid (98:2, *v/v*) and dried using a SpeedVac concentrator (Savant SPD121P, Thermo Scientific, Massachusetts, USA). The extracts were reconstituted with 200 μL of solvent A/B (90:10, *v/v*) (see above) (Dominguez-Perles et al., 2014).

The quantification of the analytes detected was performed using the authentic markers summarized in the 'Reagents' section. Twenty microliters of each sample were acquired in a UHPLC/MS/MS (UHPLC-1290 Series and a 6460 QqQ–MS/MS; Agilent Technologies, Waldbronn, Germany). The concentrations of the analytes were calculated using standard curves prepared freshly each day.

Table 2
Urine concentration (mg excreted during 0–12 h and 12–24 h) of the analytes in the control group and volunteers upon consumption of half and one serving of fresh broccoli sprouts.

Urine fraction	Analyte[Z]	Control	½ serving	1 serving	ANOVA *P*-value
0–12 h	GR	n.d.[Y]	n.d.	n.d.	n.s.[W]
	SFN-GSH	n.d.	0.12 ± 0.07a	0.08 ± 0.04a	n.s.
	SFN-Cys	n.d.	1.37 ± 0.38b	2.48 ± 0.42a	*
	SFN-NAC	n.d.	11.03 ± 2.31b	34.32 ± 12.00a	***
	SFN	n.d.	0.92 ± 0.34b	2.14 ± 0.16a	**
12–24 h	GR	n.d.	n.d.	n.d.	n.s.
	SFN-GSH	n.d.	0.24 ± 0.09a	0.07 ± 0.05a	n.s.
	SFN-Cys	n.d.	0.09 ± 0.05b	0.25 ± 0.08a	**
	SFN-NAC	n.d.	1.25 ± 0.51b	3.61 ± 0.47a	***
	SFN	n.d.	0.01 ± 0.00b	0.13 ± 0.01a	***

[Z] GR: glucoraphanin, SFN-GSH: sulforaphane-glutathione, SFN-Cys: sulforaphane-cysteine, SFN-NAC: sulforaphane-*N*-acetylcysteine.
[Y] Means ± SD ($n = 3$) within a row followed by a different lowe-case letter are significantly different at $P < 0.05$ according to Duncan's multiple range test. n.d.: Non-detected.
[W] n.s.: Non-significant at $P > 0.05$; *, **, *** significant at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

The GR and SFN metabolites were resolved chromatographically on a ZORBAX Eclipse Plus C-18 Rapid Resolution HD (2.1 × 50 mm, 1.8 μm) column (Agilent Technologies, Waldbronn, Germany). The column temperatures were held at 10 °C (left and right). The Multiple Reaction Monitoring (MRM) dynamic mode was performed in the positive mode. Dwell time was 30 ms for all MRM transitions. The mobile phases employed were solvent A: ammonium acetate, 13 mM (pH 4 with acetic acid) and solvent B: acetonitrile/acetic acid (99.9:0.1, *v/v*). The flow-rate was 0.3 mL min^{-1}, using the linear gradient scheme (t; %B): (0.0; 12), (0.2; 20), (1.0; 52), (2.5; 95), and (2.5; 12). The optimal ESI conditions for maximal detection of the analytes were: gas temperature, 225 °C; sheath gas temperature, 350 °C; capillary voltage, 3500 V; nozzle voltage, 1250 V; sheath gas flow, 12%; gas flow, 10; nebulizer, 40. The acquisition time was 2.5 min for each sample, with a post-run of 1.5 min for the column equilibration (Dominguez-Perles et al., 2014). Data acquisition was performed using MassHunter software, version B.04.00 (Agilent, Waldbronn, Germany). The urinary concentrations of GR and its metabolites were calculated from the area ratio of the ion peaks of the compounds to those of the corresponding standards.

The Food and Drug Administration (FDA) guidelines for bioanalytical method validation (http://www.fda.gov/cder/guidance) and the International Conference on Harmonization (ICH) guidelines, with suitable modifications, were followed for the validation assay. Fundamental parameters were determined, such as recovery, sensitivity, linearity, precision, and limits of detection and quantification.

2.5. UHPLC–QqQ–MS/MS analyses of the prostanoids

The separation of IsoPs and prostanoids in the volunteers' urine was also performed using a UHPLC/MS/MS (Agilent Technologies, Waldbronn, Germany) with the Medina et al. set-up conditions (Medina, Domínguez-Perles, Gil, et al., 2012). The eicosanoids are thoroughly glucuronidated *in vivo*. For this reason, a validated method for the hydrolysis of the glucuronidates (Medina, Domínguez-Perles, Cejuela-Anta, et al., 2012; Medina, Domínguez-Perles, Gil, et al., 2012) was used in order to obtain the total quantity of free and conjugated eicosanoids.

Twenty microliters of each sample were acquired in a UHPLC 1290 Series coupled to a 6460 QqQ–MS/MS (Agilent Technologies, Waldbronn, Germany). Data acquisition was performed using MassHunter software version B.04.00 (Agilent), and the concentrations of the analytes were calculated using standard curves of the available authentic markers, as summarized in the 'Reagents' section, freshly prepared each day.

2.6. Extraction and determination of vitamin C

The total vitamin C, as the sum of ascorbic acid (AA) and dehydroascorbic acid (DHAA), was determined by HPLC-UV in urine samples (20 μL), according to Pérez-Balibrea et al. (2008).

2.7. Statistical analyses

The quantitative data are presented as means ± SD. Specific differences between the target compound concentrations in the time-matched control urine and the urine at 0–12 h and 12–24 h following the dietary intervention (Fig. 1) were examined by a multifactorial analysis of variance (ANOVA) and a multiple range test (Duncan's test). All the statistical analyses were performed using the SPSS 19.0 software package (LEAD Technologies, Inc., Chicago, USA). The level of statistical significance was set at $P < 0.05$.

3. Results and discussion

The quantification of the GR and SFN and its mercapturic derivatives was carried out by daily preparation of calibration curves using standard solutions. Calibration curves were fitted by the linear regression equation '$y = ax + b$', the correlation coefficient (r^2) being higher than 0.99 within the correlation range of 37–1250 nmol mL^{-1} for each separate analyte. These r^2 values indicate an adequate linearity of the analytical procedure. The recovery of the target compounds, involving the lower, median, and upper concentrations of the linear range, was 93%–99%, 91%–100%, 89%–95%, 91%–97%, and 87%–98% for GR, SFN-GSH, SFN-Cys, SFN-NAC, and SFN, respectively. The sensitivity of an analytical method is the capability of the technical procedure to discriminate differences in the concentration or mass of the compounds as well as to provide a linear range indication. Thus, the LOQ was 37 nmol L^{-1}, except for SFN-GSH that exhibited a much-higher LOQ (156 nmol L^{-1}). The LOD was 4, 20, 6, 5, and 7 nmol L^{-1} for GR, SFN-GSH, SFN-Cys, SFN-NAC, and SFN, respectively. Both the precision and accuracy were within the acceptable limit (<15%) established by the International Conference on Harmonization (ICH), 1994). Thus, the CV% ranged from 0.73 to 8.32 and from 0.50 to 9.43 for the intra-day and inter-day multiple determinations, respectively. The accuracies recorded varied between 90.5% and 97.0% (intra-day) and between 89.6% and 97.8% (inter-day) (Dominguez-Perles et al., 2014).

3.1. Bioavailability of glucoraphanin, sulforaphane, and vitamin C

When analyzing the plant material, only GR and SFN were studied – their concentrations being 169.82 mg and 12.330 mg 100 g^{-1} fw,

respectively. These results indicate that volunteers consuming a half- and one serving of broccoli sprouts ingested an average of 51 mg and 102 mg of GR and 4 mg and 7 mg of SFN, respectively, more than those on the control diet. The analysis of urine samples from the two experimental groups who had eaten broccoli sprouts detected the excretion of SFN and its mercapturates, whereas GR was not found. The amount of bioactive compounds excreted was higher in the first 12 h after broccoli intake than in the following 12–24-h period. The analyte detected at the highest amount in 0–12-h urine was SFN-NAC (11.03 mg and 34.32 mg in volunteers that had an intake of 30 g and 60 g of broccoli sprouts, respectively), whereas SFN-GSH was the one with the lowest excretion ratio (Table 2). The bioavailability of GR and its metabolic derivatives reached 30% and 40%, on average, in volunteers consuming a half- and one serving of broccoli sprouts, respectively.

The determination of the vitamin C excreted in the control urine, as well as its concentration in the 0–12-h/12–24-h urines, gave a concentration of 12.23/10.08 mg in the former and a significant increase after the broccoli sprouts consumption (26.99/22.54 mg and 48.19/36.63 mg, on average, for the volunteers who ingested a half- and 1 serving of broccoli sprouts, respectively) (Fig. 2). The bioavailability calculated for vitamin C was 30% and 60%, on average, in volunteers consuming a half- or one serving of broccoli sprouts, respectively.

3.2. Evaluation of urinary IsoPs and prostanoids before and after broccoli sprouts intake

The analysis of eicosanoids allowed the detection of four IsoPs: 8-iso PGF2α, 8-iso-15(R)-PGF2α, 2,3-dinor-8-iso-PGF2α, and 2,3-dinor-11β-PGF2α; two prostaglandins: tetranor-PGEM and 11β-PGF2α; and the thromboxane 11-dehydro-TXB2 (Fig. 3). The other analyzed markers – the 8-iso-15-keto PGF2α, 9,11-dideoxy-9α,11α-methanoepoxy PGF2α (U-46619), the 9,11-dideoxy-9α,11α-epoxymethano PGF2α (U-44069), 2,3-dinor-6-keto PGF1α, 6-keto PGF1α, and tetranor-PGFM (tetranor-PGF-metabolite) – were not detected in urine. In addition, no significant variation of the total and individual IsoPs concentrations was detected after the intake of broccoli sprouts, when compared with the control urines (Fig. 4A).

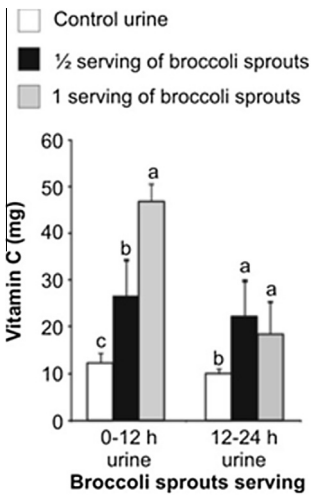

Fig. 2. Urinary vitamin C (mg 12-h^{-1}) excreted in 0–12-h and 12–24-h urines following the intake of broccoli sprouts (1 serving of broccoli sprouts, $n = 6$; or a half-serving of broccoli sprouts, $n = 6$), in comparison to the 0–12-h and 12–24-h urines of the control group ($n = 12$).

Regarding prostanoids, tetranor-PGEM, 11β-$PGF_{2\alpha}$, and 11-dehydro-TXB_2 were detected and quantified in urine in all groups. These compounds underwent a similar, significant decrease in concentration in the 0–12-h and 12–24-h periods for both doses of broccoli sprouts (Fig. 4B and C), with significant differences between the servings. The reduction of tetranor-PGEM (the main metabolite of the prostaglandins E_1 and E_2, involved in a number of physiological reactions, like inflammatory processes (Moita et al., 2013) was only significant in the first 0–12 h and the decrease ranged from 56% to 68%. The 11β-$PGF_{2\alpha}$ (a metabolite of the prostaglandin D_2 – also related with inflammatory processes (Murata et al., 2013) displayed a significant reduction in both the 0–12-h and 12–24-h urine (70% and 74% lower, respectively) in comparison to the control group urines (volunteers that did not consume Brassica foods). The same trend was found for 11-dehydro-TXB_2 (a metabolite of the thromboxane A_2 (TXA_2)) in volunteers who consumed broccoli sprouts in relation to the control: 91% and 94% decreases for the 0–12-h and the 12–24-h urines, respectively (Fig. 4C).

Currently, the influence of *Brassica* consumption on the modulation of oxidative stress events and the inflammation/vascular reactions is scarce (Fowke, Morrow, Motley, Bostick, & Ness, 2006). For this reason, combining the analysis of IsoPs and prostanoids, as markers of oxidative processes and inflammatory/vascular reactions, with the bioavailability of isothiocyanates and vitamin C could enhance our knowledge of the biological benefits associated with the consumption of broccoli sprouts.

Young, edible broccoli sprouts were selected since they are a significant dietary source of GR. We found GR concentrations that were twice those found in other reports, based on the influence of variety (origin of the seed) and the greater sensitivity of theUPHLC/MS/MS-based method (Fahey, Zhang, & Talalay, 1997; Pérez-Balibrea, Moreno, & García-Viguera, 2010; Dominguez-Perles et al., 2014) compared with glucosinolates detection by HPLC-DAD.

Previous reports have shown the urinary excretion (24-h urine) of 30 mg L^{-1}, on average, after the intake of a half-serving of fresh broccoli sprouts. In our study, the excretion was 13.44 mg L^{-1} for SFN and its mercapturic conjugates in the 0–12-h urine of volunteers that consumed a half-serving of broccoli sprouts (which corresponds to 44.8% of the previously-reported value) (Clarke et al., 2011a). However, a previous report describing the bioavailability of these compounds after broccoli sprouts intake is in accordance with our results (Dominguez-Perles et al., 2014). Differences between previous reports may be due to the distinct contents of GR and SFN in the broccoli sprouts used in the different assays, which are dependent on a plethora of agronomic and environmental factors (Pérez-Balibrea, Moreno, & García-Viguera, 2011). Our data suggest that the bioavailability of SFN was dose-dependent; one serving of fresh broccoli sprouts increased significantly the bioavailability, by up to 40%. Previous reports have shown a bioavailability of SFN ranging from 24% to 74% (Cramer & Jeffery, 2011; Vermeulen, Van Den Berg, Freidig, Van Bladeren, & Vaes, 2006), which may have been influenced by other phytochemicals and non-nutrients present in the food matrix, the co-ingestion of other foods, and the genetic predisposition according to the availability of appropriate enzymes (mainly glutathione-*S*-transferases) (Clarke et al., 2011a). Additionally, the high rate of interconversion to erucin (an isothiocyanate formed from the glucosinolate glucoerucin) could have reduced the amount of SFN after metabolization and masked the real data regarding bioavailability. The nature of the food matrix may also contribute to the difference in the SFN bioavailability when considering different serving sizes (Clarke et al., 2011a; Clarke et al., 2011b; Pérez-Balibrea et al., 2011).

The consumption of a half- or one serving of broccoli sprouts increased, in a dose related manner, the excreted vitamin C by 150%, on average (Fig. 2). The consumption of the 60-g serving of

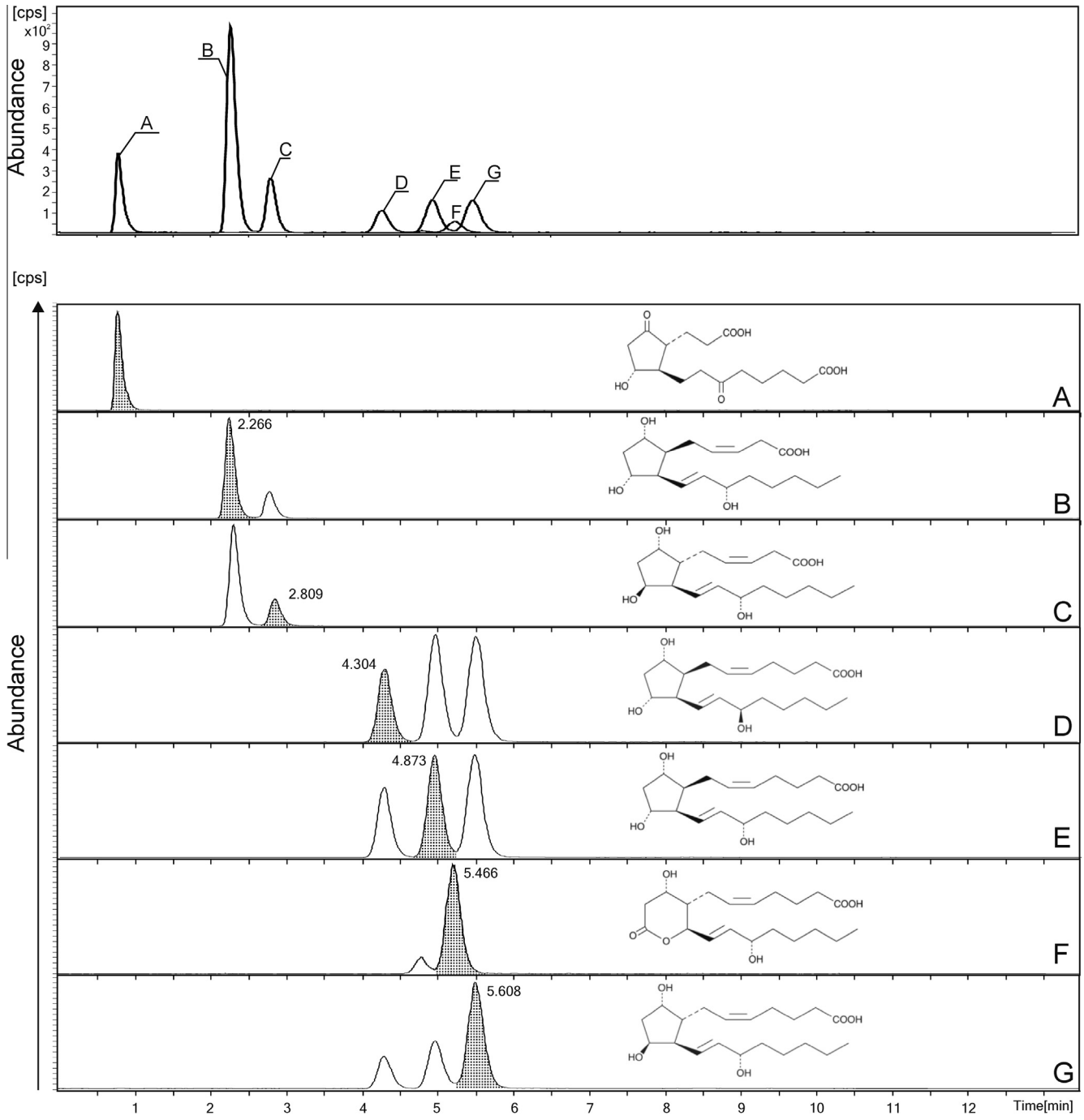

Fig. 3. UHPLC–MS/MS chromatograms of the eicosanoids detected in volunteers who participated in the study. (A) Tetranor-PGEM, (B) 2,3-dinor-8-iso-$PGF_{2\alpha}$, (C) 2,3-dinor-11β-$PGF_{2\alpha}$, (D) 8-iso-15(R)-$PGF_{2\alpha}$, (E) 8-iso-$PGF_{2\alpha}$, (F) 11-dehydro-TXB_2, (G) 11β-$PGF_{2\alpha}$.

broccoli sprouts would be enough to achieve 97.3% of the recommended dietary allowance of vitamin C (Pérez-Balibrea et al., 2011).

When analyzing the concentration of eicosanoids in urine and their modifications due to the dietary intervention, the oxidative stress-related isoprostanes (8-iso-$PGF_{2\alpha}$, 8-iso-15(R)-$PGF_{2\alpha}$, 2,3-dinor-8-iso-$PGF_{2\alpha}$, and 2,3-dinor-11β-$PGF_{2\alpha}$) and prostanoids responsible of the inflammation and vascular reactions (tetranor-PGEM (metabolite of the prostaglandins E_1 and E_2), 11β-$PGF_{2\alpha}$ (main metabolite of the prostaglandin D_2), and 11-dehydro-TXB_2 (metabolite of the thromboxane B_2, marker of the *in vivo* thromboxane A_2 synthesis)), were found. The data obtained were in the range previously reported by Medina, Domínguez-Perles, Gil, et al. (2012) in healthy volunteers (with the exception of the undetected tetranor-PGFM).

Healthy volunteers consuming broccoli sprouts exhibited no significant variation in the concentration of oxidative stress-related IsoPs in urine in comparison with controls (Fig. 4A). Although a previous report suggested a reduction in the urinary concentrations of oxidative stress-related IsoPs, as a consequence of cruciferous food intake after four weeks of dietary intervention, the reduction was not statistically significant (Fowke et al., 2006).

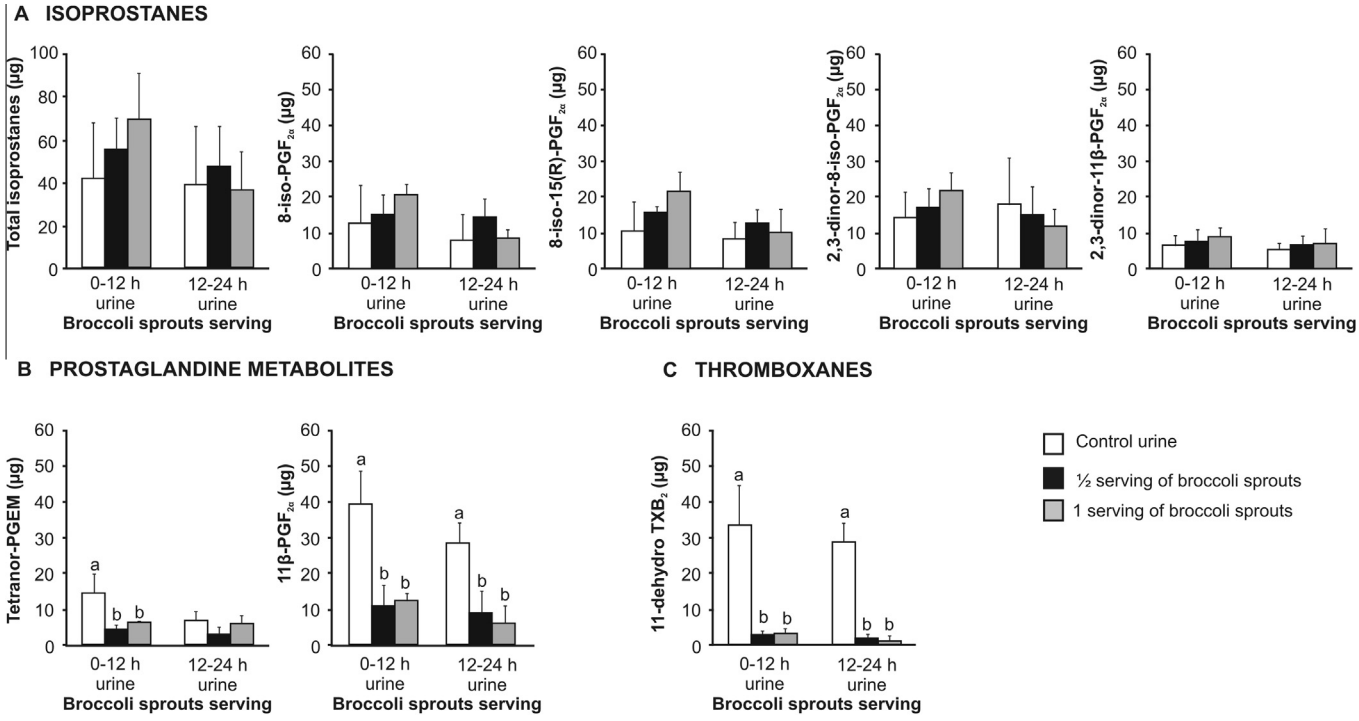

Fig. 4. Quantitative analysis of the total and single oxidative stress-related isoprostanes: 8-iso $PGF_{2\alpha}$, 8-iso-15(R)-$PGF_{2\alpha}$, 2,3-dinor-8-iso-$PGF_{2\alpha}$, and 2,3-dinor-11β-$PGF_{2\alpha}$ (A), inflammation-related prostaglandins metabolites: tetranor-PGEM and 11β-$PGF_{2\alpha}$ (B), and vascular disease-related thromboxanes metabolites: 11-dehydro-TXB_2 (C) (µg 12 h^{-1}), determined in volunteers that consumed a half-serving ($n = 6$) or one serving ($n = 6$) of broccoli sprouts – in comparison to the 0–12-h and 12–24-h urines of the control group ($n = 12$). Different lower-case letters indicate significant differences between separate dietary interventions and the time-matched control urine at $P < 0.05$, according to Duncan's multiple range test.

In this study, the single-dose intervention did not affect the synthesis and excretion of the individual and total IsoPs. This suggests a need for longer intervention periods, to obtain more robust support for the findings found previously regarding oxidative stress reactions. Moreover, our data *in vivo* contradict previous reports showing the potential of the bioactive phytochemicals from broccoli sprouts to inhibit oxidative reactions *in vitro* (Pérez-Balibrea et al., 2011). This is important because IsoPs are useful biomarkers of oxidative stress *in vivo*, and they resist the functional activity of dietary bioactive compounds better than other biomarkers, such as 4-hydroperoxy-2-nonenal (HPNE) metabolites (Kuiper, Bruno, Traber, & Stevens, 2011).

Both the half- and full servings of broccoli sprouts were able to reduce specifically the excretion of PGs and TXs in a similar proportion, in both the 0–12-h and 12–24-h urine fractions. The PGs and TXs have been related to inflammation and vascular reactions *in vivo* (Davies, Bailey, Goldenberg, & Ford-Hutchinson, 1984; Funk, 2001). Cyclooxygenase-2 (COX-2) is up-regulated in inflammation and cancer processes, and is responsible for the synthesis of PGs from arachidonic acid; therefore, it catalyzes an increase in the synthesis of PGs during the course of these processes (Zhou, Joplin, Cross, & Templeton, 2012). The reduction of the COX-2 activity constitutes a suitable way to decrease the synthesis of some PGs, and hence a potential anti-inflammatory therapy (Surh & Na, 2008). In addition, inflammation and cancer processes share molecular characteristics and pathways related to the eukaryotic redox-sensitive transcription factor (NF-κB), which is involved in the modulation of phosphorylation and other physiological reactions (Surh & Na, 2008). Our data regarding the bioavailability of SFN and its relationship with the urinary concentration of prostanoids show a significant inverse correlation between the SFN bioavailability and the urinary tetranor-PGEM and 11-dehydro-TXB_2 (−0.699, $P < 0.001$ and −0.459, $P < 0.05$, respectively, in both the 0–12-h and 12–24-h periods), whereas the reduction of 11β-$PGF_{2\alpha}$ showed no significant correlation with SFN bioavailability. This suggests that the modulation of the biological secretion of 11β-$PGF_{2\alpha}$ is influenced by SFN – even though this action may be further modulated by distinct compounds present in broccoli sprouts, such as phenolic compounds. This is in agreement with previous reports on the biological activity of ITCs through the inhibition of COX-2, IL-1β, TNF_{α}, and Nfr2 expression, in the realm of inflammatory processes (Lin et al., 2008).

The consumption of a half- or one serving of broccoli sprouts caused an increase in the vitamin C content of urine. There was no correlation between vitamin C bioavailability and changes in the IsoPs synthesis and excretion *in vivo*, in agreement with previous work (Levine, Wang, Padayatty, & Morrow, 2001). Oxidative stress is a complex phenomenon that may be influenced by multiple variables, such as the selection of antioxidants or their dose to effectively suppress the oxidative stress. Moreover, when analyzing the relationships between the bioavailability of vitamin C and the metabolism of prostanoids, we observed that the amount of vitamin C and the amounts of tetranor-PGEM, 11β-$PGF_{2\alpha}$, and 11-dehydro TXB_2 excreted in the urine showed an inverse relationship, in agreement with Sánchez-Moreno et al. (2004). This indicates that vitamin C reduces the formation of compounds derived from the phospholipids oxidation through inhibition of the COX-2 enzymatic activity (Jiang et al., 2012; Sánchez-Moreno et al., 2004).

4. Conclusion

Analysis of the bioavailability of sulforaphane and vitamin C, and correlation with the modification of the synthesis and

excretion of eicosanoids related to oxidative stress, inflammation, and vascular reactions, showed that sulforaphane and vitamin C were associated with changes in the urinary concentrations of compounds linked to inflammation and vascular reactions whereas the effect on oxidative stress metabolism *in vivo* was almost absent. These results reinforce the previous data on the effect of isothiocyanates on inflammatory processes, the molecular pathways of which are partially shared by tumoral processes. So, the consumption of this healthy food should be a successful and valuable tool in the control of metabolic processes associated with chronic diseases whose clinical courses are marked by inflammatory and vascular reactions.

Acknowledgements

This study was supported by the projects AGL2011-23690 (CICYT) and CSD007-0063 (CONSOLIDER-INGENIO 2010 'Fun-C-Food') and by the Fundación Séneca (Comunidad Autónoma de la Región de Murcia, 'Group of Excellence in Research' 04486/GERM/06). Sonia Medina Escudero and Raúl Domínguez-Perles were appointed under a CICYT Research Contract (AGL2011-23690) and a CSIC Research Contract, respectively. We thank to Dr. David Walker for the revision of the English language.

References

Blatnik, M., & Steenwyk, R. C. (2010). Quantification of urinary PGEm, 6-keto PGF1α and 2,3-dinor-6-keto PGF1α by UFLC–MS/MS before and after exercise. *Prostaglandins & Other Lipid Mediators, 93*(1–2), 8–13.

Clarke, J. D., Dashwood, R. H., & Ho, E. (2008). Multi-targeted prevention of cancer by sulforaphane. *Cancer Letters, 269*(2), 291–304.

Clarke, J. D., Hsu, A., Riedl, K., Bella, D., Schwartz, S. J., Stevens, J. F., et al. (2011a). Bioavailability and inter-conversion of sulforaphane and erucin in human subjects consuming broccoli sprouts or broccoli supplement in a cross-over study design. *Pharmacological Research, 64*(5), 456–463.

Clarke, J. D., Riedl, K., Bella, D., Schwartz, S. J., Stevens, J. F., & Ho, E. (2011b). Comparison of isothiocyanate metabolite levels and histone deacetylase activity in human subjects consuming broccoli sprouts or broccoli supplement. *Journal of Agricultural and Food Chemistry, 59*(20), 10955–10963.

Comporti, M., Signorini, C., Arezzini, B., Vecchio, D., Monaco, B., & Gardi, C. (2008). Isoprostanes and hepatic fibrosis. *Molecular Aspects of Medicine, 29*(1–2), 43–49.

Cracowski, J. L., Tremel, F., Marpeau, C., Baguet, J. P., Stanke-Labesque, F., Mallion, J. M., et al. (2000). Increased formation of F2-isoprostanes in patients with severe heart failure. *Heart, 84*(4), 439–440.

Cramer, J. M., & Jeffery, E. H. (2011). Sulforaphane absorption and excretion following ingestion of a semi-purified broccoli powder rich in glucoraphanin and broccoli sprouts in healthy men. *Nutrition and Cancer, 63*(2), 196–201.

Davies, P., Bailey, P. J., Goldenberg, M. M., & Ford-Hutchinson, A. W. (1984). The role of arachidonic acid oxygenation products in pain and inflammation. *Annual Reviews in Immunology, 2*, 335–357.

Dinkova-Kostova, A. T., & Kostov, R. V. (2012). Glucosinolates and isothiocyanates in health and disease. *Trends Molecular Medicine, 18*(6), 337–347.

Dominguez-Perles, R., Medina, S., Moreno, D. T., García-Viguera, C., Ferreres, F., & Gil-Izquierdo, T. (2014). A new ultra-rapid UHPLC/MS/MS method for assessing glucoraphanin and sulforaphane bioavailability in human urine. *Food Chemistry, 143*, 132–138.

Fahey, J. W., Zhang, Y., & Talalay, P. (1997). Broccoli sprouts: An exceptionally rich source of inducers of enzymes that protect against chemical carcinogens. *Proceedings of the National academy of Sciences of the United States of America, 94*(19), 10367–10372.

Food and Drug Administration (FDA) (2001) US Department of Health and Human Services. *Guidance for Industry: Bioanalytical method validation*. http://www.fda.gov/cder/guidance.

Fowke, J. H., Morrow, J. D., Motley, S., Bostick, R. M., & Ness, R. M. (2006). Brassica vegetable consumption reduces urinary F2-isoprostane levels independent of micronutrient intake. *Carcinogenesis, 27*(10), 2096–2102.

Funk, C. D. (2001). Prostaglandins and leukotrienes: Advances in eicosanoid biology. *Science, 294*(5548), 1871–1875.

Holt, E. M., Steffen, L. M., Moran, A., Basu, S., Steinberger, J., Ross, J. A., et al. (2009). Fruit and vegetable consumption and its relation to markers of inflammation and oxidative stress in adolescents. *Journal of the American Dietetic Association, 109*(3), 414–421.

International Conference on Harmonization (ICH) (1994). Validation of analytical method. *Definitions and terminology*. Geneva: ICH Q2A.

Jiang, H., Harrison, F. E., Jain, K., Benjamin, S., May, J. M., Graves, J. P., et al. (2012). Vitamin C activation of the biosynthesis of epoxyeicosatrienoic acids. *Advances in Bioscience and Biotechnology, 3*, 204–218.

Kaviarasan, S., Muniandy, S., Qvist, R., & Ismail, I. S. (2009). F2-isoprostanes as novel biomarkers for type 2 diabetes: A review. *Journal of Clinical Biochemistry and Nutrition, 45*(1), 1–8.

Kuiper, H. C., Bruno, R. S., Traber, M. G., & Stevens, J. F. (2011). Vitamin C supplementation lowers urinary levels of 4-hydroperoxy-2-nonenal metabolites in humans. *Free Radical Biology & Medicine, 50*(7), 848–853.

Levine, M., Wang, Y., Padayatty, S. J., & Morrow, J. (2001). A new recommended dietary allowance of vitamin C for healthy young women. *Proceedings of the National academy of Sciences of the United States of America, 98*(17), 9842–9846.

Lin, W., Wu, R. T., Wu, T., Khor, T. O., Wang, H., & Kong, A. N. (2008). Sulforaphane suppressed LPS-induced inflammation in mouse peritoneal macrophages through Nrf2 dependent pathway. *Biochemical Pharmacology, 76*(8), 967–973.

Medina, S., Domínguez-Perles, R., Cejuela-Anta, R., Villaño, D., Martínez-Sanz, J. M., Gil, P., et al. (2012). Assessment of oxidative stress markers and prostaglandins after chronic training of triathletes. *Prostaglandins & Other Lipid Mediators, 99*(3–4), 79–86.

Medina, S., Domínguez-Perles, R., Gil, J. I., Ferreres, F., García-Viguera, C., Martínez-Sanz, J. M., et al. (2012). A ultra-pressure liquid chromatography/triple quadrupole tandem mass spectrometry method for the analysis of 13 eicosanoids in human urine and quantitative 24 h values in healthy volunteers in a controlled constant diet. *Rapid Communications in Mass Spectrometry, 26*(10), 1249–1257.

Milatovic, D., Montine, T. J., & Aschner, M. (2011). Measurement of isoprostanes as markers of oxidative stress. *Methods in Molecular Biology, 758*, 195–204.

Moita, E., Gil-Izquierdo, A., Sousa, C., Ferreres, F., Silva, L. R., Valentão, P., et al. (2013). Integrated analysis of COX-2 and iNOS derived inflammatory mediators in LPS-stimulated RAW macrophages pre-exposed to *Echium plantagineum* L. Bee pollen extract. *PLoS ONE, 8*(3).

Morrow, J. D., Hill, K. E., Burk, R. F., Nammour, T. M., Badr, K. F., & Roberts Ii, L. J. (1990). A series of prostaglandin F2-like compounds are produced in vivo in humans by a non-cyclooxygenase, free radical-catalyzed mechanism. *Proceedings of the National Academy of Sciences of the United States of America, 87*(23), 9383–9387.

Morrow, J. D., Minton, T. A., Mukundan, C. R., Campbell, M. D., Zackert, W. E., Daniel, V. C., et al. (1994). Free radical-induced generation of isoprostanes in vivo. Evidence for the formation of D-ring and E-ring isoprostanes. *Journal of Biological Chemistry, 269*(6), 4317–4326.

Morrow, J. D., Zackert, W. E., Yang, J. P., Kurhts, E. H., Callewaert, D., Dworski, R., et al. (1999). Quantification of the major urinary metabolite of 15-F(2T)-isoprostane (8-iso-PGF(2α)) by a stable isotope dilution mass spectrometric assay. *Analytical Biochemistry, 269*(2), 326–331.

Murata, T., Aritake, K., Tsubosaka, Y., Maruyama, T., Nakagawa, T., Hori, M., et al. (2013). Anti-inflammatory role of PGD2 in acute lung inflammation and therapeutic application of its signal enhancement. *Proceedings of the National academy of Sciences of the United States of America, 110*(13), 5205–5210.

Pérez-Balibrea, S., Moreno, D. A., & García-Viguera, C. (2008). Influence of light on health-promoting phytochemicals of broccoli sprouts. *Journal of the Science of Food and Agriculture, 88*(5), 904–910.

Pérez-Balibrea, S., Moreno, D. A., & García-Viguera, C. (2010). Glucosinolates in broccoli sprouts (*Brassica oleracea* var. *italica*) as conditioned by sulphate supply during germination. *Journal of Food Science, 75*(8), C673–C677.

Pérez-Balibrea, S., Moreno, D. A., & García-Viguera, C. (2011). Genotypic effects on the phytochemical quality of seeds and sprouts from commercial broccoli cultivars. *Food Chemistry, 125*(2), 348–354.

Pérez-Sala, D. (2011). Electrophilic eicosanoids: Signaling and targets. *Chemico-Biological Interactions, 192*(1–2), 96–100.

Roberts Ii, L. J., & Morrow, J. D. (1997). The generation and actions of isoprostanes. *Biochimica et Biophysica Acta – Lipids and Lipid Metabolism, 1345*(2), 121–135.

Sánchez-Moreno, C., Cano, M. P., De Ancos, B., Plaza, L., Olmedilla, B., Granado, F., et al. (2004). Consumption of high-pressurized vegetable soup increases plasma vitamin C and decreases oxidative stress and inflammatory biomarkers in healthy humans. *Journal of Nutrition, 134*(11), 3021–3025.

Speid, L. (2010). *Clinical trials: what patients and volunteers need to know* (1st ed.). USA: Oxford University Press Inc.

Surh, Y. J., & Na, H. K. (2008). NF-κB and Nrf2 as prime molecular targets for chemoprevention and cytoprotection with anti-inflammatory and antioxidant phytochemicals. *Genes and Nutrition, 2*(4), 313–317.

Vermeulen, M., Van Den Berg, R., Freidig, A. P., Van Bladeren, P. J., & Vaes, W. H. J. (2006). Association between consumption of cruciferous vegetables and condiments and excretion in urine of isothiocyanate mercapturic acids. *Journal of Agricultural and Food Chemistry, 54*(15), 5350–5358.

West, L. G., Meyer, K. A., Balch, B. A., Rossi, F. J., Schultz, M. R., & Haas, G. W. (2004). Glucoraphanin and 4-hydroxyglucobrassicin contents in seeds of 59 cultivars of broccoli, raab, kohlrabi, radish, cauliflower, brussels sprouts, kale, and cabbage. *Journal of Agricultural and Food Chemistry, 52*(4), 916–926.

Zhou, J., Joplin, D. G., Cross, J. V., & Templeton, D. J. (2012). Sulforaphane inhibits prostaglandin E2 synthesis by suppressing microsomal prostaglandin E synthase 1. *PLoS ONE, 7*(11), e49744.