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ORIGINAL ARTICLE

Determination of new biomarkers to monitor the dietary consumption of isothiocyanates

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Abstract

Isothiocyanates (ITCs) found in cruciferous vegetables have been associated with a reduced cancer risk in humans. We determined serum albumin adducts of allyl isothiocyanate (AITC), benzylisothiocyanate (BITC), phenylethylisothiocyanate (PEITC) and sulforaphane (SFN) in 85 healthy men from a dietary, randomized, controlled trial. After enzymatic digestion of albumin we determined the adducts of the ITCs with lysine (Lys) using liquid chromatography–tandem mass spectrometry. At the beginning of the study (and after 4 weeks) 4.7% (2.4%), 48.2% (35.3%), 5.9% (10.6%), and 24.7% (23.5%) of the samples were found positive for AITC-Lys, BITC-Lys, PEITC-Lys and SFN-Lys, respectively. This method enables the quantification of ITC adducts in albumin from large, prospective studies on diet and cancer.

Keywords: *Chemical carcinogenesis; mass spectroscopy; biomonitoring; protein adducts; chemoprevention*

Introduction

Glucosinolates (GLs) occur mainly in cruciferous vegetables (Fahey et al. 2001, Rosa et al. 1997). Isothiocyanates (ITCs) are released from GLs by the enzyme myrosinase (Figure 1), when tissues of raw plants are chewed (Bones & Rossiter 2006, Holst & Williamson 2004). ITCs have demonstrated cancer preventive activity in animals, and increased dietary intake of ITCs has been shown to be associated with a reduced cancer risk in humans (Higdon et al. 2007, Hecht 2000). Steinbrecher et al. (2009) showed an inverse association between dietary intake of GLs and the risk of prostate cancer. ITCs exert their cancer chemopreventive action by modulating the activities of phase I and phase II drug metabolism enzymes (Sparnins et al. 1982, Maheo et al. 1997, Zhang et al. 1992, Hecht 2000, von Weymarn et al. 2006). ITCs and their thiol conjugates inhibit the cell cycle and cause apoptotic cell death (Mi et al. 2007, 2008). ITC conjugates with thiols are reversible (Brusewitz et al. 1977, Jiao et al. 1996). It has been shown with radiolabelled phenethylisothiocyanate (PEITC) and sulforaphane (SFN) that the initial conjugation predominantly occurs with cellular GSH. With increasing time, protein binding becomes the major reaction, at least in part because of dissociation of ITC from the unstable adducts of ITC with the thiol group of GSH (Mi et al. 2007). The time course of this protein binding correlated well with the inhibition of proliferation and the induction of apoptosis. This finding suggests that cellular protein adducts of ITC may be an early event for apoptosis induction. Therefore biomarkers are needed which show the presence of stable reaction products with proteins.

Animal studies have shown that GSH conjugates are the major products of ITCs (reviewed in Conaway et al. 2002, Zhang 2004). *N*-Acetyl cysteine conjugates of ITCs have been used as a urinary biomarker of exposure to dietary GLs (reviewed in Zhang 2004) in humans. Most epidemiological studies on the relationship between diet and cancer have relied on information collected with questionnaires monitoring food intake. This is known not to be accurate (Jenab et al. 2009). Urinary metabolites might provide the exposure history of the last 24h, if the urine of the full next day is collected. However, this is not

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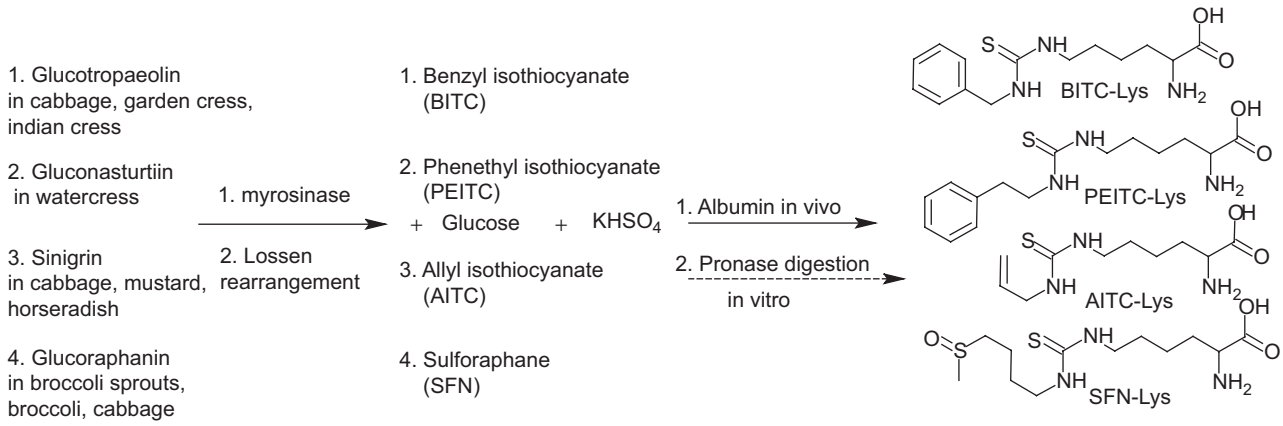

Figure 1. Release of isothiocyanates *in vivo* and formation of adducts with albumin.

feasible in large epidemiological studies. Therefore, stable and unreactive biomarkers are needed, which reflect a larger time span of the ITC-exposure history. Thus, we developed a method to determine (not cysteine adducts) reaction products of ITCs with albumin in humans (Kumar & Sabbioni 2010) (Figure 1). The PEITC and benzylisothiocyanate (BITC) albumin adducts yielded a half-life of 21–23.2 days, which was close to the values of the half-life of albumin of 20–25 days (Skipper & Tannenbaum 1990). Protein adducts might be involved in the chemopreventive effects of ITCs (Mi et al. 2009). Therefore, blood protein adducts are a potential surrogate marker for the effects of ITCs at the cellular level. Albumin adducts of ITCs were found in a human subject given a large dose of cruciferous vegetables (Kumar & Sabbioni 2010). In the present study we tested the applicability of the method in a group of healthy men not exposed to specific ITC-rich diets. The results of this study will indicate the feasibility of the method for large, prospective studies on diet and cancer.

Materials and methods

Subjects

In the randomized controlled trial Overall Dietary Supplementation Study (Malaveille et al. 1998, Guarrera et al. 2007), 85 healthy heavy smokers of air-cured tobacco were recruited among Italian blood donors. Trial participants were all male, aged 35–70 years, resident in the Torino metropolitan area (northern Italy), healthy on the basis of a medical questionnaire. All the volunteers gave written informed consent according to ethical standard requirements. Participants were randomly assigned to three groups, corresponding to three different diets: group A (n=29), the diet was rich in flavonoids, but not supplemented;group B (n =29), the diet was a normal iso-caloric diet with an adequate administration of fruit and vegetables; and group C (n=27), the diet was based on supplementation of the normal diet with additional flavonoids in the form of green tea and soy products. The participants were taught how to prepare food for the three different diets during a six-lesson course with a professional cook. The volunteers followed the prescribed diet for 4 weeks. All participants filled in a validated food frequency questionnaire (FFQ) (Pisani et al. 1997) at the beginning of the study and then started filling in a daily diary during the experimental month. The baseline data are a recall of the diet intake from the previous year. Finally a FFQ was filled in again 1 year after the end of the trial. Intake of cruciferous vegetables (cabbage, kale, broccoli, broccoflower, cauliflower,brussel sprouts, land cress, turnip root, radish and mustard seeds) were estimated through a self-administrated daily diary, checked weekly and abstracted from a dietician.

Biological samples

Non-fasting blood (20 ml) was collected at the beginning of the study (set 0) and after 4 weeks (set 4). The blood samples were processed, separated in aliquots of plasma and buffy coats and stored at -20°C on the day of collection.

Chemicals

Methanol and acetonitrile for liquid chromatography–tandem mass spectrometry(LC-MS/MS) was obtained from Fisher Scientific (Morris Plains, NJ, USA). Pronase E from *Streptomyces griseus* (cat. no. 81748), formic acid (MS grade, no. 94318) and ammonium formate (cat. no. 17843) were purchased from Fluka (Buchs, Switzerland). Human serum albumin (cat. no. A1653) and water for LC-MS/MS (Chromasolv; cat. no. 39253) were purchased from Sigma-Aldrich (St Louis, MO, USA). Chromabond C18ec cartridge (500 mg per 3 ml) was purchased from Macherey-Nagel (Düren, Germany). N^6-[(Allylamino]

carbonothioyl]lysine (AITC-Lys), N^6-[(benzylamino) carbonothioyl]lysine (BITC-Lys), N^6-{[(2-phenylethyl) amino]carbonothioyl}lysine (PEITC-Lys), N^6-({[3-(methylsulfinyl)propyl]amino}carbonothioyl)lysine (SFN-Lys), and the corresponding isotope-labelled standards,AITC–[$^{13}C_6$ $^{15}N_2$]Lys, BITC–[$^{13}C_6$ $^{15}N_2$]Lys, PEITC–[$^{13}C_6$ $^{15}N_2$]Lys, and SFN–[$^{13}C_6$ $^{15}N_2$]Lys, were obtained as described previously (Kumar & Sabbioni 2010).

Instrumentation

API 4000 Q Trap (Applied Biosystems, Foster City, CA, USA) mass spectrometer interfaced to a HPLC (Shimadzu Prominance 20AD) was used for the LC-MS/MS analyses. A UV-1800 spectrophotometer from Shimadzu (Kyoto, Japan) was used for protein determination. Centrifugations were performed on a Beckman Coulter Allegra™X-22R centrifuge equipped with a SX4250 swing out bucket rotor (Beckman Coulter, Fullerton, CA, USA).

Albumin isolation

Albumin was isolated from thawed pure plasma (0.4 ml) by dropwise addition of an equal volume of saturated $(NH_4)_2SO_4$ (Troester et al. 2000). This mixture was centrifuged at 9000*g* to remove immunoglobulins. The supernatant was dialysed using Eppendorfcups with an integrated 10kDa molecular cut-off filter (Pall Life Sciences (Ann Arbor, MI, USA) centrifugal devices for biomolecular separations, P/N 0D010C35). The tubes were centrifuged for 40 min at 9500*g*. The residue was taken up in 500 µl of water and centrifuged again for 30 min. Samples were redissolved in 10 mM sodium phosphate buffer (pH 7.4). The concentrations of the isolated albumin solutions were determined with a Coomassie protein Assay kit (cat. no. 23236) for total protein quantitation from Pierce (Thermo Fisher Scientific, Rockford, IL, USA).

Albumin digestion

Albumin (9 mg) was incubated with Pronase E (3 mg) in sodium bicarbonate (50 mM, 2 ml, pH 8.9) 37°C overnight in the presence of the isotope-labelled standards: BITC-[$^{13}C_6$ $^{15}N_2$]Lys (1.61 pmol), PEITC-[$^{13}C_6$ $^{15}N_2$]Lys (1.57 pmol), AITC-[$^{13}C_6$ $^{15}N_2$]Lys (19.7 pmol) and SFN-[$^{13}C_6$ $^{15}N_2$] Lys (15.1 pmol). The digested samples were acidified (up to pH 4.0) with 2 M hydrochloric acid and applied to Chromabond C18ec columns for solid-phase extraction. The columns were activated with methanol (3 ml) and then equilibrated with 0.1% formic acid (3 ml, pH 4.0). The samples were applied on the column and then washed with 0.1% formic acid (3 ml, pH 4.0). BITC-Lys, PEITC-Lys, SFN-Lys and AITC-Lys were eluted with 80% methanol in 0.1% formic acid (6 ml, pH 4.0). After evaporation to approximately 1 ml in a speed evacuator, an aliquot 25–75 µl was analysed by LC-MS/MS.

LC-MS/MS method for the determination of BITC–Lys, PEITC–Lys, AITC–Lys and SFN–Lys

LC-MS/MS optimization

Shimadzu Prominance 20AD interfaced to a API 4000 Q Trap LC-MS/MS (Applied Biosystems) system was used for all the quantitative analyses. The MS parameters were optimized in the electrospray ionization mode (ESI) with 100 pg ml^{-1} solutions of the analytes and a flow rate of 10 µl min^{-1}. The parameters were optimized in the positive ionization mode for SFN-Lys (*m/z* 324.1 $[M + H]^+$), and in the negative ionization mode for BITC-Lys (*m/z* 294.0 $[M - H]^-$), PEITC-Lys (*m/z* 308.1 $[M - H]^-$) and AITC-Lys (*m/z* 244.1 $[M - H]^-$). The quadrupole mass analysers (Q1 and Q3) were set at a 0.7 ± 0.1 amu resolution window.

The MS was operated with an electrospray voltage at -4500 V for negative ionization and +5500V for positive ionization and with a source temperature of 500°C. Nitrogen was used as ion spray (GS1), drying (GS2) and curtain gas at 40, 45 and 10 arbitrary units, respectively. The declustering potential (DP) and collision energy (CE) for BITC-Lys, PEITC-Lys, AITC-Lys and SFN-Lys were -45, -50, -40, +61 and -24, -24, -25, +23 V, respectively. The entrance potential (EP) for all compounds was set at -10 V. All data were processed using Analyst software 1.4.2 (Applied Biosystems/MDS Sciex).

BITC-Lys, PEITC-Lys, AITC-Lys and SFN-Lys were detected with multiple reaction monitoring (MRM) transition of 294.0/145.0, 308.1/145.0, 244.1/145.0 $[M - H]^-$ and 324.1/136.0 $[M + H]^+$, respectively, along with their corresponding stable isotope-labelled compounds as internal standards: BITC-[$^{13}C_6$ $^{15}N_2$]Lys {302.1/153.0 $[M - H]^-$}, PEITC-[$^{13}C_6$ $^{15}N_2$]Lys {316.1/153.0 $[M - H]^-$}, AITC-[$^{13}C_6$ $^{15}N_2$]Lys {252.1/153.0 $[M - H]^-$ }, SFN-[$^{13}C_6$ $^{15}N_2$]Lys {332.1/136.0 $[M + H]^+$}.

The chromatographic separation of BITC-Lys, PEITC-Lys and AITC-Lys was performed on a Luna 3 µmC18(2) (100 Å, 150 × 2.0 mm, 3 µm) with a C18 guard column (AJO-4287; 4 mm L × 3.0 mm ID) (Phenomenex Inc., Torrance, CA, USA), using 10 mM ammonium formate (solvent A) and acetonitrile (solvent B) at a flow rate of 0.2 ml min^{-1}. The gradient program was: 0.10 min (B 0%), 3 min (B 0%) and 16 min (B 80%). The retention time (t_R) of BITC-Lys, PEITC-Lys and AITC-Lys was 12.7, 13.1 and 11.1 min, respectively. The column flow was diverted away from the ESI ion source except for the time period from 8 to 18 min. SFN-Lys was analysed on the same column and the same solvent system but with a slightly different gradient and with positive ESI-MS detection. The gradient program was: 0.10 min (B 0%), 3 min (B 0%) and 17 min (B 80%). The retention time (t_R) of SFN-Lys was 11.5 min. The

column flow was diverted away from the ESI ion source except for the time period from 8 to 13 min.

Calibration line

To generate the calibration line, human serum albumin (9 mg) was spiked with different amount of BITC-Lys (0.00, 0.21, 0.85, 1.69, 3.39, 16.94 and 33.89 pmol), PEITC-Lys (0.00, 0.20, 0.80, 1.61, 3.23, 16.17 and 32.35 pmol), AITC-Lys (0.00,0.25, 1.01, 2.03, 4.07 and 20.39 pmol) and SFN-Lys (0.00, 0.19, 0.77, 1.54, 3.09, 15.47 and 30.95 pmol) along with the corresponding isotope-labelled compounds: BITC-[$^{13}C_6{}^{15}N_2$]Lys (1.61 pmol), PEITC-[$^{13}C_6{}^{15}N_2$]Lys (1.57 pmol), AITC-[$^{13}C_6{}^{15}N_2$]Lys (19.7 pmol) and SFN-[$^{13}C_6{}^{15}N_2$]Lys (15.1 pmol). The samples were digested and worked up as described above. The calibration lines for BITC-Lys, PEITC-Lys, AITC-Lys and SFN-Lys were generated over the range of 0.00–33.89, 0.00–32.35, 0.00–20.39 and 0.00–30.95 pmol per 9 mg albumin, respectively. The concentration levels of BITC-Lys, PEITC-Lys, AITC-Lys and SFN-Lys were plotted against the peak area ratio of BITC-Lys/BITC-[$^{13}C_6{}^{15}N_2$-Lys], PEITC-Lys/PEITC-[$^{13}C_6{}^{15}N_2$-Lys], AITC-Lys/AITC-[$^{13}C_6{}^{15}N_2$-Lys] and SFN-Lys/SFN-[$^{13}C_6{}^{15}N_2$-Lys], respectively. The regression coefficients $r^2 = 0.999$ (BITC-Lys), 0.998 (PEITC-Lys), AITC-Lys (0.998) and SFN-Lys (0.999) were obtained using the regression option, linear and 1/x weighting factor. The limit of quantification (LOQ) for BITC-Lys, PEITC-Lys and AITC-Lys following this work-up and analysis procedure was 5.33, 4.12 and 14.4 fmol mg^{-1} albumin, respectively. The limit of detection (LOD) for BITC-Lys, PEITC-Lys and AITC-Lys analysed with LC-MS/MS was 1.69, 1.61 and 4.80 fmol (on column), respectively. The LOQ for SFN-Lys following this work-up and analysis procedure was 15.4 fmol mg^{-1} albumin. The LOD for SFN-Lys analysed with LC-MS/MS was 1.54 fmol (on column). The signal/noise ratio for the LOD and LOQ determination was >10.

Statistical analyses

The statistical analyses were performed with SPSS 16.0 (SPSS Inc., Chicago, IL, USA). The data were not normally distributed (one-sample Kolmogorov–Smirnov test, $p<0.05$). Therefore, non-parametric tests were used for correlations (Spearman-rank correlations), for the comparison of groups (Mann–Whitney test) and for the comparison of paired samples (Wilcoxon sign test).

Results

For the present study we determined the albumin adducts of ITCs in a group of people not on a specific cruciferous vegetables diet. The analytical method for the determination of albumin adducts of ITCs was published recently (Kumar & Sabbioni 2010). This method was modified and applied to samples collected in a diet and nutrition study (Guarrera et al. 2007, Malaveille et al. 2004). Albumin was isolated from the plasma after ammonium sulfate precipitation and digested with Pronase E. The digests were analysed with LC-MS/MS. The released single amino acid adducts of AITC, BITC, PEITC and SFN with lysine were quantified using isotope dilution mass spectrometry. AITC-Lys, BITC-Lys, PEITC-Lys and SFN-Lys present in the biological samples were quantified against the corresponding isotope-labelled internal standard AITC-[$^{13}C_6{}^{15}N_2$]Lys, BITC-[$^{13}C_6{}^{15}N_2$]Lys, PEITC-[$^{13}C_6{}^{15}N_2$]Lys and SFN-[$^{13}C_6{}^{15}N_2$]Lys.

The results were shown in Tables 1–3 and Figure 2. The samples (n=85) were collected at the beginning (set 0) of the monitored diet study and after 4 weeks (set 4). First we analysed the results obtained for the two time points without considering the diet subgroups (see below). At the beginning of the study (and after 4 weeks), 4.7% (2.4%), 48.2% (35.3%), 5.9% (10.6%) and 24.7% (23.5%) of the samples were found positive for AITC-Lys, BITC-Lys, PEITC-Lys and SFN-Lys, respectively. The 95th percentile adduct levels found at the beginning of the study decreased in the following order: SFN-Lys (239 fmol mg^{-1}) > AITC-Lys (104 fmol mg^{-1}) > BITC-Lys (13.1 fmol mg^{-1}) > PEITC-Lys (6.8 fmol mg^{-1}). At the end of the study the 95th percentile adduct levels decreased in the following order: SFN-Lys (187 fmol mg^{-1}) > BITC-Lys (11.2 fmol mg^{-1}) > PEITC-Lys (8.3 fmol mg^{-1}) > AITC-Lys (0 fmol mg^{-1}). Therefore the adduct levels of SFN-Lys were up to 33 times higher than for BITC-Lys and PEITC-Lys. The adduct levels were not significantly different between the samples collected at the two time points. The relationship between the different adducts were analysed using Spearman rank correlations. At the beginning of the study only the adducts AITC-Lys and SFN-Lys correlated

Table 1. Spearman rank correlations r (p= two-tailed significance level) between the adduct levels found in the subjects after 4 weeks of monitored diet.

	AITC-Lys	BITC-Lys	PEITC-Lys
BITC-Lys	0.238 (0.028)		
PEITC-Lys	0.474 (<0.001)	0.104 (0.342)	
SFN-Lys	0.328 (0.002)	0.205 (0.060)	0.480 (0.001)

AITC, allyl isothiocyanate; BITC, benzylisothiocyanate; PEITC, phenylethylisothiocyanate; SFN, sulforaphane; Lys, lysine.

Table 2. Spearman rank correlations r (p= two-tailed significance level) between the adduct levels found after 4 weeks with the total cruciferous vegetables intake monitored for 4 weeks.

	AITC-Lys	BITC-Lys	PEITC-Lys	SFN-Lys
Total cruciferous vegetables (4 weeks)	0.306 (0.016)	0.190 (0.14)	0.403 (0.001)	0.399 (0.001)

AITC, allyl isothiocyanate; BITC, benzylisothiocyanate; PEITC, phenylethylisothiocyanate; SFN, sulforaphane; Lys, lysine.

Table 3. The levels of BITC-Lys and SFN-Lys and the total cruciferous vegetable levels determined with a questionnaire were compared among the three diet groups and the two different time points (beginning of the study = set 0, and after 4 weeks= set 4). The significant differences found between two groups using the Mann–Whitney test were marked with a pair of equal numbers 1–8: [1–6]$p<0.05$; and [7,8]$p<0.1$. In addition the percentage of subjects found positive for all adducts (AITC-Lys, BITC-Lys, PEITC-Lys, SFN-Lys) are listed.

Set	Group (*n*)	AITC-Lys (%)	BITC-Lys (%)	PEITC-Lys (%)	SFN-Lys (%)	BITC-Lys (fmolmg^{-1}), median (25th,75th,90th)	SFN-Lys (fmol mg^{-1}), median, (25th,75th,90th)	Total cruciferous vegetables (g daily), median (25th,75th,90th)
0	A (*n*=29)	3.4	69	10.3	13.8	5.39[1,7] (0, 7.07, 7.98)	0[3](0,0, 71.7)	4.9[8] (3.5, 8.7, 12.2)
4	A (*n*=29)	6.9	34.5	17.2	31	0[7] (0, 6.18, 8.30)	0(0, 25.2, 172)	68.8 [5] (29.3, 110, 145)
0	B (*n*=29)	6.9	55.2	0	17.2	5.86 [2](0, 8.26, 12.7)	0 [4](0, 0, 98.9)	2.4 (*n*=17)[8] (1.5, 8.9, 12.7)
4	B (*n*=29)	0	37.9	0	10.3	0(0, 7.95, 11.3)	0(0,0, 38.7)	6.3 (*n*=17)[5,6] (3.0, 16.7, 32.7)
0	C (*n*=27)	3.7	18.5	7.4	44.4	0 [1,2](0, 0, 9.56)	0 [3,4](0, 43.3, 196)	4.5 (*n*=16) (2.0, 8.7, 18.1)
4	C (*n*=27)	0	33.3	14.8	29.6	0 (0, 5.74, 9.05)	0 (0, 21.3, 169)	38.7 (*n*=16) (26.5, 73.4, 109)[6]

AITC,allyl isothiocyanate; BITC, benzylisothiocyanate; PEITC, phenylethylisothiocyanate; SFN, sulforaphane; Lys, lysine.

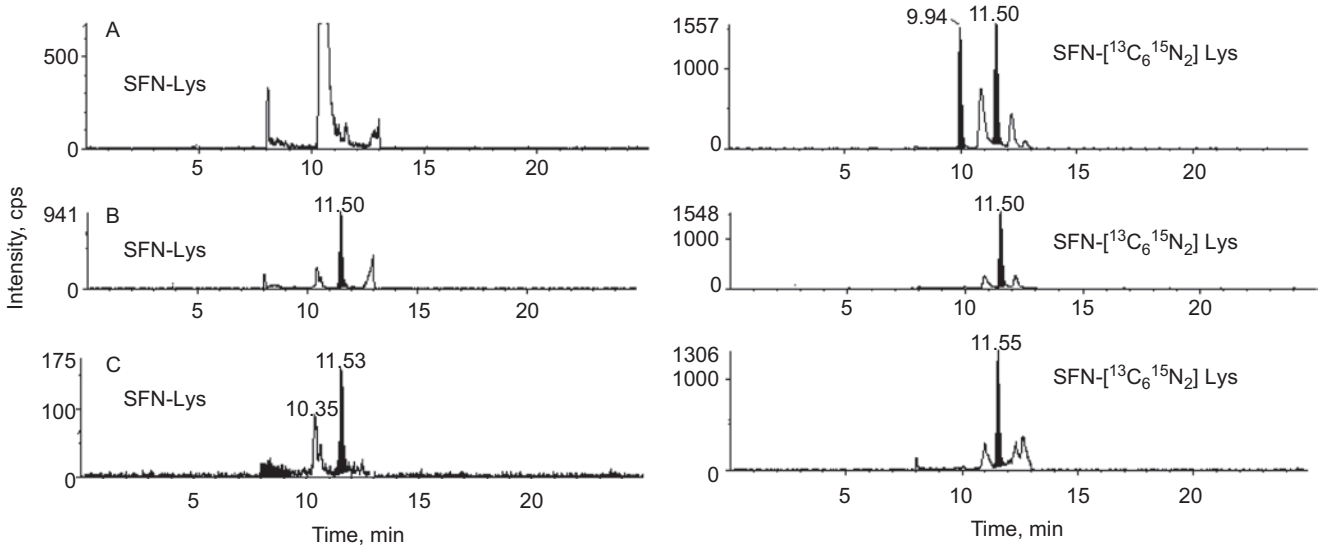

Figure 2. Liquid chromatography–tandem mass spectrometry (LC-MS/MS) analyses of albumin digest obtained from: (A) a human sample without sulforaphane(SFN)-lysine (Lys); (B) a human sample with SFN-Lys; and (C) an analytical standards of SFN-Lys. The impurity at 10.4 min in panel A does not interfere with the peak of SFN-Lys at 11.5 min.

(0.277, p=0.010) with each other. After 4 weeks, except for the BITC-Lys levels, all the other adducts levels (AITC-Lys, PEITC-Lys and SFN-Lys) correlated with each other (Table 1). The same correlations were performed for the adduct levels between the two time points. Only the SFN-Lys adducts found at the two time points correlated significantly with r=0.345 (p=0.001). The adduct levels were compared with the diet data collected and published previously (Guarrera et al. 2007, Malaveille et al. 2004). The following values were found for the total cruciferous vegetable consumption per day for the baseline data (FFQ) (2.0, 4.5, 8.9, 12.0 g daily =25th, 50th, 75th, 95th percentile) and for the data collected during 4 weeks (daily diary) (16.0, 34.0, 79.4, 144 g daily). The baseline diet data did not correlate with any of the measured ITC-Lys adducts (data not shown). In Table 2 we compared the total cruciferous vegetable data monitored for 4 weeks with the adduct levels. Except for the BITC-Lys adduct levels, all other adduct levels measured after 4 weeks correlated with the food intake. The food intake data were compared between the two time points. The total cruciferous vegetable consumptions recorded during the 4 weeks were significantly higher than the baseline data according to the Mann–Whitney test for two independent samples or the Wilcoxon signed rank test for two related samples. Therefore one would expect significantly higher adduct levels after 4 weeks. However this was not the case. Probably the baseline data are not as accurate as the food intake data collected daily for 4 weeks.

The subjects were classified in three groups, according to their dietary intake. Therefore, in total there are six groups: 0-A, 0-B, 0-C and 4-A, 4-B, 4-C (Table 3). The adduct levels were not significantly different between the two time points according to the Mann–Whitney test for two independent samples or the Wilcoxon signed rank test for two related samples. However, at the beginning of the study the adduct levels of BITC-Lys and/or SFN-Lys were significantly different between group A, C and the groups B and C (Mann–Whitney test), although the assignment of the subjects to the groups was randomized (Table 3). After 4 weeks the adduct levels of the three groups were not significantly different. During the 4 weeks of the study the diet intake was closely monitored. This might have influenced the dietary intake of subjects, which then yielded similar adduct levels in the groups.

The food baseline data were not significantly different among the three diet categories A–C (Table 3). In contrast, significant differences were seen among the diet categories after 4 weeks. The levels in group B were significantly lower than in group A and group C. In summary, it appears that group B was eating a smaller amount of cruciferous vegetables. This explains the development of the SFN-Lys adduct levels. After 4 weeks, group B had the lowest amount of positive samples for SFN-Lys adducts. This seems not be the case for the BITC-Lys adducts, where after 4 weeks the amount of positive samples is similar in all three groups. BITC is present in garden cress which is consumed as salad; in addition BITC is present in condiments (Vermeulen et al. 2006). Garden cress was not consumed by the subjects; however the condiments have not been studied in detail for the present study.

Discussion

This is the first study measuring ITC adducts of blood proteins in a population. In the past, exposure was estimated from questionnaires and from the urinary levels of mercapturic acids (see Introduction). The population of the present study was not exposed specifically to a diet rich in GLs but rich in flavonoids. The purpose of the present analyses was to show the feasibility of the new method in a random group of people.

The presence of BITC reaction products as the most prevalent ITC-exposure marker in the present subjects is surprising according to dietary information obtained from the participants of this study, asglucoraphanin (precursor of SFN) and sinigrin (precursor of AITC), are present in more vegetables than glucotropaeolin (precursor of BITC) and gluconasturtiin (precursor of PEITC) (Steinbrecher & Linseisen 2009, McNaughton & Marks 2003). Therefore, one would expect more positive samples for SFN and AITC. However in a cohort collected for the European Prospective Investigation into Cancer and Nutrition (EPIC)-Heidelberg study, Steinbrecher and Linseisen (2009) estimated a 1.46 ± 1.27, 1.01 ± 0.19, 3.41 ± 0.48, 4.25 ± 0.48 μmol daily consumption of glucotropaeolin (precursor of BITC), gluconasturtiin (precursor of PEITC), glucoraphanin (precursor of SFN) and sinigrin (precursor of AITC), respectively. Therefore according to these estimates the intake differences among the different ITCs are small. In a 70-kg man there would be ~100 g of albumin. Assuming that 0.1–1% of the daily dose would form BITC adducts with albumin, we would obtain 1.46–14.6 nmol BITC-Lys per 100 g albumin or 14.6–146 fmol mg^{-1} adduct. For chronically exposed people, the steady-state adduct level would be 29 times higher. This would yield 423.4–4234 fmol mg^{-1}adduct. The actual adduct levels were 0–38.6 fmol mg^{-1}. AITC and SFN are expected to be present to a larger extent in the food samples. Most samples were negative for AITC–adducts. SFN–adducts were found only in <25% of the subjects. This might be for different reasons. The developed assay is approximately three times more sensitive for BITC adducts, although the detection limit (compound on column) was similar for all compounds. In addition the adduct yields after uptake or ITCs is not known. In order to improve the sensitivity of the assay, ITC–antibodies should be produced to construct immunoaffinity columns for the purification of the digests. The ion-suppressing matrix could be reduced in such a manner, which then would improve the sensitivity of the assay.

In conclusion, the poor correlation of the baseline diet data with the adduct levels at the beginning of the study reflects the difficulty in collecting dietary data in retrospect (recall of the last 12 month). In contrast, the directly recorded diet data during the 4-week experiment correlated significantly with the adduct levels, except for the BITC-adduct levels.

Our study provides proof-of-principle that albumin adducts of ITCs are a potentially useful biomarker for the intake of ITCs in epidemiological studies, overcoming the limitations of questionnaire estimates. Epidemiological studies that have found a protective effect of ITCs for lung cancer (Brennan et al. 2005, London et al. 2000) were based on urine measurements, which, however, reflect a short time intake and metabolism of the compounds. The longer half-life of albumin adducts allows the use of this markers as an integrated measurement over weeks.

Declaration of interest

We are most grateful for the financial support of the Tulane Cancer Center. The authors report no conflicts of

interest. The authors alone are responsible for the content and writing of the paper.

References

Bones AM, Rossiter JT. (2006). The enzymic and chemically induced decomposition of glucosinolates.*Phytochemistry* 67:1053–67.

Brennan P, Hsu CC, Moullan N, Szeszenia-Dabrowska N, Lissowska J, Zaridze D, Rudnai P, Fabianova E, Mates D, Bencko V, Foretova L, Janout V, Gemignani F, Chabrier A, Hall J, Hung RJ, Boffetta P, Canzian F. (2005). Effect of cruciferous vegetables on lung cancer in patients stratified by genetic status: a mendelian randomisation approach. *Lancet* 366:1558–60.

Brusewitz G, Cameron BD, Chasseaud LF, Gorler K, Hawkins DR, Koch H, Mennicke WH. (1977). The metabolism of benzyl isothiocyanate and its cysteine conjugate. *Biochem J* 162:99–107.

Conaway CC, Yang YM, Chung FL. (2002). Isothiocyanates as cancer chemopreventive agents: their biological activities and metabolism in rodents and humans. *Curr Drug Metab* 3:233–55.

Fahey JW, Zalcmann AT, Talalay P. (2001). The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. *Phytochemistry* 56:5–51.

Guarrera S, Sacerdote C, Fiorini L, Marsala R, Polidoro S, Gamberini S, Saletta F, Malaveille C, Talaska G, Vineis P, Matullo G. (2007). Expression of DNA repair and metabolic genes in response to a flavonoid-rich diet. *Br J Nutr* 98:525–33.

Hecht SS. (2000). Inhibition of carcinogenesis by isothiocyanates. *Drug Metab Rev* 32:395–411.

Higdon JV, Delage B, Williams DE, Dashwood RH. (2007). Cruciferous vegetables and human cancer risk: epidemiologic evidence and mechanistic basis. *Pharmacol Res* 55:224–36.

Holst B, Williamson G. (2004). A critical review of the bioavailability of glucosinolates and related compounds.*Nat Prod Rep* 21:425–47.

Jenab M, Slimani N, Bictash M, Ferrari P, Bingham SA. (2009). Biomarkers in nutritional epidemiology: applications, needs and new horizons. *Hum Genet* 125:507–25.

Jiao D, Conaway CC, Wang MH, Yang CS, Koehl W, Chung FL. (1996). Inhibition of N-nitrosodimethylaminedemethylase in rat and human liver microsomes by isothiocyanates and their glutathione, L-cysteine, and N-acetyl-L-cysteine conjugates. *Chem Res Toxicol* 9:932–8.

Kumar A, Sabbioni G. (2010). New biomarkers for monitoring the levels of isothiocyanates in humans. *Chem Res Toxicol* 23:756–65.

London SJ, Yuan JM, Chung FL, Gao YT, Coetzee GA, Ross RK, Yu MC. (2000). Isothiocyanates, glutathione S-transferase M1 and T1 polymorphisms, and lung-cancer risk: a prospective study of men in Shanghai, China. *Lancet* 356:724–9.

Maheo K, Morel F, Langouet S, Kramer H, Le Ferrec E, Ketterer B, Guillouzo A. (1997). Inhibition of cytochromes P-450 and induction of glutathione S-transferases by sulforaphane in primary human and rat hepatocytes. *Cancer Res* 57:3649–52.

Malaveille C, Fiorini L, Bianchini M, Davico L, Bertinetti S, Allegro G, Hautefeuille A, Sacerdote C, Vineis P. (2004). Randomized controlled trial of dietary intervention: association between level of urinary phenolics and anti-mutagenicity. *Mutat Res* 561:83–90.

Malaveille C, Hautefeuille A, Pignatelli B, Talaska G, Vineis P, Bartsch H. (1998). Antimutagenic dietary phenolics as antigenotoxic substances in urothelium of smokers.*Mutat Res* 402:219–24.

McNaughton SA, Marks GC. (2003). Development of a food composition database for the estimation of dietary intakes of glucosinolates, the biologically active constituents of cruciferous vegetables. *Br J Nutr* 90:687–97.

Mi L, Gan N, Cheema A, Dakshanamurthy S, Wang X, Yang DC, Chung FL. (2009). Cancer preventive isothiocyanates induce selective degradation of cellular (alpha)- and (beta)-tubulins by proteasomes. *J Biol Chem* 284:17039–51.

Mi L, Wang X, Govind S, Hood BL, Veenstra TD, Conrads TP, Saha DT, Goldman R, Chung FL. (2007). The role of protein binding in induction of apoptosis by phenethyl isothiocyanate and sulforaphane in human non-small lung cancer cells. *Cancer Res* 67:6409–16.

Mi L, Xiao Z, Hood BL, Dakshanamurthy S, Wang X, Govind S, Conrads TP, Veenstra TD, Chung FL. (2008). Covalent binding to tubulin by isothiocyanates: a mechanism of cell growth arrest and apoptosis. *J Biol Chem* 283:22136–146.

Pisani P, Faggiano F, Krogh V, Palli D, Vineis P, Berrino F. (1997). Relative validity and reproducibility of a food frequency dietary questionnaire for use in the Italian EPIC centres. *Int J Epidemiol* 26 (Suppl. 1):S152–60.

Rosa EAS, Heaney RK, Fenwick GR, Portas CAM. (1997). Glucosinolates in crop plants. *Hortic Rev* 19:99–215.

Skipper PL, Tannenbaum SR. (1990). Protein adducts in the molecular dosimetry of chemical carcinogens. *Carcinogenesis* 11:507–18.

Sparnins VL, Venegas PL, Wattenberg LW. (1982). Glutathione S-transferase activity: enhancement by compounds inhibiting chemical carcinogenesis and by dietary constituents. *J Natl Cancer Inst* 68:493–6.

Steinbrecher A, Linseisen J. (2009). Dietary intake of individual glucosinolates in participants of the EPIC-Heidelberg cohort study.*Ann Nutr Metab* 54:87–96.

Steinbrecher A, Nimptsch K, Husing A, Rohrmann S, Linseisen J. (2009). Dietary glucosinolate intake and risk of prostate cancer in the EPIC-Heidelberg cohort study. *Int J Cancer* 125:2179–86.

Troester MA, Lindstrom AB, Kupper LL, Waidyanatha S, Rappaport SM. (2000). Stability of hemoglobin and albumin adducts of benzene oxide and 1,4-benzoquinone after administration of benzene to F344 rats. *Toxicol Sci* 54:88–94.

Vermeulen M, van den Berg R, Freidig AP, van Bladeren PJ, Vaes WH. (2006). Association between consumption of cruciferous vegetables and condiments and excretion in urine of isothiocyanate mercapturic acids.*J Agric Food Chem* 54:5350–8.

vonWeymarn LB, Chun JA, Hollenberg PF. (2006). Effects of benzyl and phenethyl isothiocyanate on P450s 2A6 and 2A13: potential for chemoprevention in smokers. *Carcinogenesis* 27:782–90.

Zhang Y. (2004). Cancer-preventive isothiocyanates: measurement of human exposure and mechanism of action. *Mutat Res* 555:173–90.

Zhang Y, Talalay P, Cho CG, Posner GH. (1992). A major inducer of anticarcinogenic protective enzymes from broccoli: isolation and elucidation of structure. *Proc Natl Acad Sci U S A* 89:2399–403.