Total Isothiocyanate Contents in Cooked Vegetables Frequently Consumed in Singapore

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The degradation products of glucosinolates in cruciferous vegetables, organic isothiocyanates (ITCs), inhibit carcinogenesis in numerous animal models including cancers of the lung, esophagus, forestomach, colon, mammary gland, and pancreas. To assist investigations aimed at understanding the role of dietary ITCs in the protection of human cancers, we examined ITC contents in various cooked vegetables frequently consumed by Singapore Chinese, whose cancer profile is distinct from that of U.S. whites (e.g., much lower rates of cancers of the breast, colon, rectum, and pancreas). Using an HPLC method to quantify the cyclic product of ITCs and 1,2-benzenedithiol after treatment of vegetable juices with myrosinase, we analyzed a total of 102 vegetable specimens. The results show considerable variation in ITC contents in the nine types of cruciferous vegetables analyzed, ranging from 4.9 μmol/100 g wet weight in bok choi (*Brassica chinensis*) to 81.3 μmol/100 g wet weight in watercress. ITCs were not detected in any of the noncruciferous vegetables examined. ITCs also were not detected in most of the cooked cruciferous vegetables prior to myrosinase treatment, indicating that only glucosinolates are present in these cooked vegetables. This study demonstrated the usefulness of the cyclic condensation reaction of ITCs with a dithiol reagent for quantifying total ITC contents in vegetables and the potential application of the assay in epidemiologic studies.

Keywords: *Isothiocyanate; cooked vegetables; Singapore*

INTRODUCTION

Natural organic isothiocyanates (ITCs) are among the degradation products of glucosinolates under the catalysis of myrosinase (thioglucoside glucohydrolase [EC 3.2.3.1]). Glucosinolates are widely distributed in cruciferous vegetables that are consumed by humans, such as broccoli, cabbage, cauliflower, radish, turnip, and watercress (VanEtten and Tookey, 1979; Fenwick et al., 1983; Daxenbichler et al., 1991). It has long been recognized that ITCs have diverse biological effects due to their chemical reactivity (Nishie and Daxenbichler, 1980; Fenwick et al., 1983). More recently, ITCs have been studied for their effects in cancer prevention. A number of natural and synthetic ITCs have been demonstrated to be potent inhibitors of tumorigenesis in various animal models [for reviews, see Chung (1992) and Hecht (1995)]. The cancer protective effect has been attributed to the ability of ITCs to inhibit phase I enzymes that are responsible for the bioactivation of carcinogens (Guo et al., 1992) and to induce phase II detoxification enzymes (Sparnins et al., 1982). Similar effects on phase I and II enzymes have been observed in humans given a large quantity of cruciferous vegetables in the diet (Hecht et al., 1995; Bogaads et al., 1994; Nijhoff et al., 1995). Also, several epidemiological studies have provided suggestive evidence that consumption of cruciferous vegetables may be associated with a reduced cancer risk (Negri et al., 1990; Steinmetz et al., 1991; Rodgers et al., 1993; Willett, 1994). Nonetheless, the exact role of ITCs in human cancer prevention remains to be defined.

Due to growing interest in the potential protective effects of dietary ITC in human cancer development, a database of glucosinolate and total ITC contents in various cruciferous vegetables from specific geographic regions is essential for assessing dietary ITC exposure in epidemiologic studies. A large number of cruciferous vegetables are consumed after cooking, and the amounts of glucosinolates are usually reduced considerably in cooked vegetables (Betz and Fox, 1994); thus, it is important to determine the levels of ITCs in cooked vegetables that have been prepared in the usual ways by the studied populations. Recently, a spectroscopic assay was described for measuring organic ITCs after reaction with vicinal dithiols (Zhang et al., 1992, 1996). Using this reaction, in this study we developed a modified, convenient, and highly quantitative HPLC-based assay that allowed us to determine total ITC contents in 102 cooked vegetable specimens comprising nine types of cruciferous vegetables and seven types of noncruciferous vegetables that are frequently consumed by Singapore Chinese whose intake of vegetables, especially green vegetables, is high.

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EXPERIMENTAL PROCEDURES

Materials. Sinigrin was purchased from Sigma Chemical Co. (St. Louis, MO). 1,2-Benzenedithiol was obtained from Lancaster Synthesis, Inc. (Windham, NH). 1,3-Benzodithiole-2-thione was prepared and characterized previously in this laboratory according to a published method (Zhang et al., 1992; Chung et al., 1998). All other chemicals were reagent grade from commercial sources. Myrosinase was prepared from mustard seeds using a published procedure (Appelqvist and Josefsson, 1967).

Instruments. An HPLC system consisting of two Shimadzu LC-10AS pumps, a SPD-10AV UV–Vis detector, a SIL-10A autoinjector, and a SCL-10A system controller (Shimadzu Scientific Instruments, Inc., Columbia, MD) was used in all analyses. An Eppendorf CH-30 column heater and a TC-50 temperature controller (Eppendorf Scientific, Inc., Madison, WI) were used to keep column temperature constant for overnight and batch analyses. A water bath shaker Julabo UC-8A (Julabo USA, Kutztown, PA) was used in all incubations. Centrifugation was done on a Savant Speed-Vac SVC-100H (Savant Instruments, Inc., Holbrook, NY).

Sample Source and Collection. Three samples of nine types each of cruciferous vegetables were purchased from various markets in Singapore on each of three different dates, which spanned a period of 4 months (November 1995, January 1996, and March 1996). The nine types of cruciferous vegetables were broccoli, cabbage, cauliflower, choi sum (*Brassica chinensis* var. *parachinensis*, also known as Chinese flowering cabbage), kai choi (*Brassica juncea* var. *rugosa*, also known as mustard cabbage or Chinese mustard), kai lan (*Brassica alboglabra*, also known as Chinese kale), bok choi (*Brassica chinensis*, also known as Chinese white cabbage), watercress, and wong nga pak *(Brassica pekinensis* var. *cylindrica*, also known as celery cabbage). Twenty-one samples of noncruciferous vegetables comprising seven types (i.e., three samples per type) also were collected from the same markets in October 1996. These included Chinese lettuce, head lettuce, green beans, spinach, snow peas, ung choi (*Ipomoea aquatica,* also known as water spinach), and yin choi (*Amaranthus tricolor*, also known as Chinese spinach). A total of 150 g of each sample of fresh vegetables was cooked in boiling water for 3 min as would be done by the studied population within 5 h after purchase. The cooked vegetable specimens were each sealed in a labeled double plastic bag and mailed on dry ice by Express Air to the American Health Foundation in Valhalla, NY. All specimens were received frozen and were stored at −20 °C until analysis.

Sample Preparations. Specimens of vegetables were allowed to thaw at room temperature. To simulate the exposure to glucosinolates for humans through ingesting cooked vegetables, the vegetables were blended with water into a fine paste to obtain the vegetable juice (Scheme 1). The content of specimen in the bag was weighed and mixed with 100 mL of deionized water. The mixture was blended for 1 min into a fine paste using a kitchen miniblender. The paste was wrapped with six layers of cheesecloth and squeezed to release the juice into a large beaker. The residue of vegetable paste was mixed with another portion of deionized water (125 mL) and was squeezed again to collect the juice as described above. The process was repeated with an additional 125 mL of water, all extracts of the vegetable juice were combined, and the volume was brought up to 500 mL with deionized water. Ten milliliters of the final vegetable juice was filtered through a filter paper under vacuum to obtain a clear filtrate.

Myrosinase-Catalyzed Release of Isothiocyanates from Vegetable Juice Filtrates. The glucosinolates in the juice were hydrolyzed by semipurified myrosinase to release ITCs (Scheme 2a). In a capped 7-mL glass vial, 1.0 mL of myrosinase solution (2.0 mg/mL in 0.1 M potassium phosphate buffer, pH 6.6) and 1.0 mL of the vegetable juice filtrate were mixed by vortex. The mixture was incubated for 2 h at 37 °C in a waterbath shaker. The reaction time of myrosinase and glucosinolate depends on the amounts of myrosinase added and glucosinolates present in the vegetable. The time period

Scheme 1. Procedures for Analysis of ITCs in Cooked Vegetables
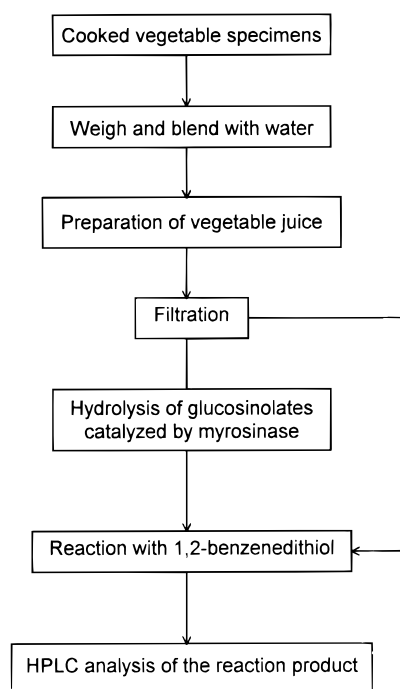
of 2 h was determined by examining the time-dependent formation of ITC from aqueous solutions of sinigrin (0.2 and 0.4 mM, much higher concentrations than those present in vegetable specimens) under the same condition as stated above. The conversion of sinigrin to allyl isothiocyanate was greater than 98% within 2 h.

HPLC Assay of Total ITC. A previously described method for urinary ITC analysis was used in this study (Chung et al., 1998). In brief, 100 μL of the above reaction mixture was mixed with 600 μL of 1,2-benzenedithiol in 2-propanol (10 mM, degassed) and 500 μL of phosphate buffer (0.1 M, pH 8.5, degassed) in a 2-mL Chromacol autosampler vial with a screw cap (Chromacol Inc., Trumbull, CT). Duplicate samples were prepared for each vegetable specimen. They were incubated at 65 °C for 2 h and centrifuged for 20 min. Sample vials were loaded to an autosampler and analyzed by HPLC. A Phenomenex Bondaclone C18 column (150 × 3.9 mm) together with a guard column C18 (30 × 3.9) (Phenomenex, Torrance, CA) was used. The mobile phase consisted of MeOH (70%) and H_2O (30%) running at a flow rate of 1.75 mL/min with a sample injection volume of 20 μL and a detection wavelength at 365 nm. A close agreement of total ITC levels was obtained for the duplicate samples, and their average values were used. The process is depicted in Scheme 1.

Statistical Analysis. The two-way analysis of variance method (Snedecor and Cochran, 1967) was used to compare total ITC levels across the three shipments and the nine types of cruciferous vegetables. All p values quotes are two-sided.

RESULTS AND DISCUSSION

A total of 102 specimens comprising nine types of cruciferous and seven types of noncruciferous vegetables were analyzed in this study. The levels of ITCs in cooked vegetables before and after myrosinase treatment were determined using a cyclocondensation reaction of ITCs with 1,2-benzenedithiol, which led to a single product 1,3-benzenedithiole-2-thione detected at 365 nm on HPLC (Scheme 2b and Figure 1). The HPLC-based method has also been recently applied in the analysis of human urine for the quantification of ITC metabolites (Chung et al., 1998). The means of ITC
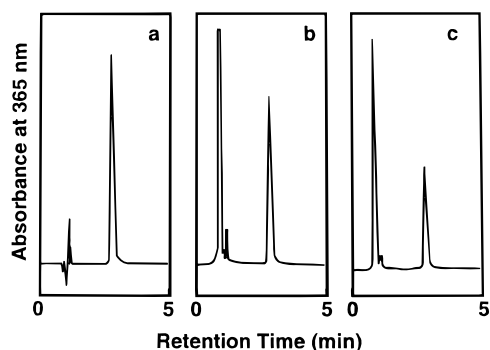
Figure 1. HPLC chromatograms of 1,3-benzenedithiole-2-thione derived from (a) synthetic standard, (b) cooked vegetable juice filtrate after myrosinase treatment, and (c) sinigrin after myrosinase treatment. The early peak in panels b and c is 1,2-benzenedithiol.

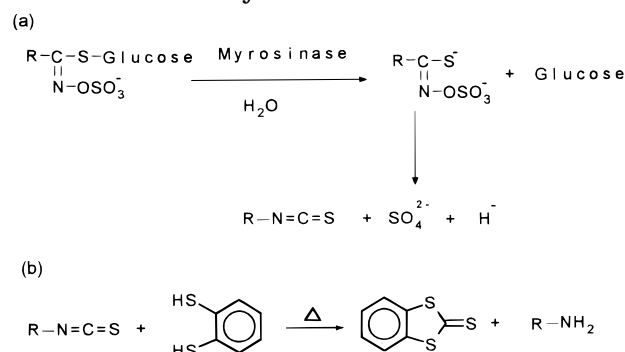**Scheme 2. Chemical and Biochemical Reactions Involved in the Analysis**

Table 1. Cruciferous and Noncruciferous Vegetables and Their Total ITC Contents

vegetables	ITC contents, mean and range (μmol/100 g wet weight)
Cruciferous Vegetables ($n = 9$)	
broccoli (*Brassica oleracea* var. *italica*)	38.6 (10.1−62.0)
cabbage (*Brassica oleracea* var. *capitata*)	27.5 (11.9−62.7)
cauliflower (*Brassica oleracea* var. *botrytis*)	11.6 (2.7−24.0)
choi sum (*Brassica chinensis* var. *parachinensis*)	11.1 (3.5−23.4)
kai choi (*Brassica juncea* var. *rugosa*)	71.2 (25.6−138.4)
kai lan (*Brassica* var. *alboglabra*)	15.4 (3.1−35.9)
bok choi (*Brassica chinensis*)	4.9 (2.0−7.5)
watercress (*Nasturtium officinale*)	81.3 (17.1−144.6)
wong nga pak (*Brassica pekinensis* var. *cylindrica*)	5.6 (2.5−8.8)
Noncruciferous Vegetables[a] ($n = 3$)	
Chinese lettuce (*Lactuca sativa*)	ND[b]
head lettuce (*Lactuca sativa*)	ND
spinach (*Spinacea oleracea*)	ND
green beans (*Phaseolus vulgaris*)	ND
snow peas (*Pisum sativum*)	ND
ung choi (*Ipomoea aquatica*)	ND
yin choi (*Amaranthus tricolor*)	ND

[a] Only one batch of noncruciferous vegetables was collected and analyzed. [b] ND, = not detected.

levels in these vegetables after myrosinase treatment and the ranges of their values are given in Table 1.

We compared total ITC levels (determined after myrosinase treatment) across the nine types of cooked cruciferous vegetables and found a highly significant difference across the nine types ($p = 0.0001$). Watercress contained the highest mean level of ITC, 81.3 μmol/100 g wet weight, while bok choi had the lowest mean level, 4.9 μmol/100 g wet weight (Table 1). The difference between the mean ITC levels of watercress and bok choi was 16-fold. On the other hand, ITC levels were not different between the three shipments for any particular type of vegetables ($p = 0.80$), suggesting little seasonal differences in total ITC contents for these vegetables in Singapore. As expected, ITC was not detected, using this procedure with a limit of detection of 0.2 pmol, in any of the noncruciferous vegetables examined in this study.

In filtered juices prepared from all 102 vegetable specimens, only three (two from kai choi and one from watercress) were found to contain detectable amounts of ITCs before myrosinase treatment. However, the amounts of ITCs present in these samples before myrosinase treatment were much lower than those released after myrosinase treatment, 0.4−0.6 versus 71.2−81.3 μmol/100 g wet weight. This is consistent with our current knowledge regarding the hydrolysis of glucosinolates by myrosinase. In fresh vegetables, myrosinases are separated from glucosinolates in plant cells (Höglund et al., 1991). When cells are damaged either by cutting or the chewing process, glucosinolates are mixed with myrosinases which catalyze the release of ITCs (Scheme 2a). Alternatively, glucosinolates may undergo thermal degradation to release ITCs when heated above 125 °C (MacLeod et al., 1981). Neither of these pathways toward release of ITCs from glucosinolates was significant under the cooking and blending conditions used in this study. This was evidenced by the nondetectable level of ITC in most vegetable juices. Clearly, myrosinases in vegetables were inactivated by cooking in boiling water for as short as 3 min. Only three samples showed low levels of ITCs, likely due to trace amounts of myrosinases that survived the heating. This also explains why cooked vegetables in general have much less pungent taste than the raw vegetables. These observations indicate that it is the glucosinolates rather than their degradation products, ITCs, in these cooked vegetables that are ingested by humans. The amount of ITCs determined after myrosinase treatment represents the maximum amount of ITCs to which humans would be exposed after consumption of these cooked cruciferous vegetables if they were fully liberated in vivo.

The large variation in ITC levels found in different types of cooked cruciferous vegetables is striking. These results illustrate the importance of acquiring specific information about each type of vegetables in epidemiologic studies in order to assess the amount of ITCs consumed by individuals. We note that total ITC contents vary considerably between samples of the same type of vegetables from different markets. This may be attributed to many factors that affect the amount of glucosinolates in vegetables such as genetic, botanical, and environmental effects (Fenwick et al., 1983). The amount of ITCs relevant to human exposure is further complicated by the method of processing and cooking the vegetables and the extent of conversion of glucosinolates to ITCs in vivo. Although the amounts of ITCs available in either cooked or raw vegetables can be quantified using the method described here, to determine human uptake of ITCs, based on these data, is still difficult due to our lack of knowledge regarding the conversion of glucosinolates to ITCs in vivo. Works from us and others have shown that more than half of the glucosinolates from raw vegetables, or allyl isothiocy-

anate in a mustard paste, consumed by humans are excreted in the urine as ITC metabolites (Mennicke et al., 1988; Chung et al., 1992; Jiao et al., 1994). While these studies demonstrate the hydrolysis of glucosinolates upon consumption of raw vegetables, questions remain as to whether glucosinolates are converted to ITCs in the human body. In a recent analysis of urine samples collected from 246 Singapore Chinese, a population known to consume only cooked cruciferous vegetables, 205 samples (83%) were found to contain ITC metabolites of various concentrations (unpublished data). Although humans can be exposed to ITCs through other sources, such as mustard or food flavoring agents, cooked cruciferous vegetables represent the major source of dietary ITCs for Singapore Chinese. It appears that most ITCs present in the urine are formed in vivo from glucosinolates in the cooked vegetables. Little is known about the process by which the human system facilitates such a transformation, although myrosinase-like enzyme activities have been found in a number of mammalian tissues and bacteria (Goodman et al., 1959; Oginsky et al., 1965). Further experiments designed to find out whether and to what extent this process occurs in humans after ingesting cooked cruciferous vegetables would allow us to assess the role of dietary ITCs as active ingredients in human health.

LITERATURE CITED

Appelqvist, L. A.; Josefsson, E. J. Method for quantitative determination of isothiocyanates and oxazolidinethiones in digests of seedmeals of rape and turnip rape. *J. Sci. Food Agric.* **1967**, *18*, 510–519.

Betz, J. M.; Fox, W. D. High-performance liquid chromatographic determination of glucosinolates in *Brassica* vegetables. In *ACS Symposium Series 546 on Food Phytochemicals for Cancer Prevention. I. Fruits and Vegetables*; Huang, M. T., Osana, T., Ho, C.-T., Rosen, R. T., Eds.; American Chemical Society: Washington, DC, 1994; pp 181–191.

Bogaads, J.; Verhagen, H.; Willems, M. L.; van Poppel, G.; van Bladeren, P. J. Consumption of brussels sprouts results in elevated α-class glutathione S-transferase levels in human blood plasma. *Carcinogenesis* **1994**, *15*, 1073–1075.

Chung, F-L. Chemoprevention of lung carcinogenesis by aromatic isothiocyanates. In: Wattenberg, L., Lipkin, M., Boone, C. W., Kelloff, G. J., Eds.; *Cancer Chemoprevention*; CRC Press: Boca Raton, FL, 1992; pp 227–245.

Chung, F.-L.; Morse, M. A.; Eklind, K. I.; Lewis, J. Quantitation of human uptake of the anticarcinogen phenethyl isothiocyanate after a watercress meal. *Cancer Epidemiol. Biomarkers Prev.* **1992,** *1*, 383–388.

Chung, F−L.; Jiao, D.; Yu, M. C. A urinary biomarker for total uptake of dietary isothiocyanates in humans. *Cancer Epidemiol. Biomarkers Prev.* **1998**, in press.

Daxenbichler, M. E.; Spencer, G. F.; Carlson, D. G.; Rose, G. B.; Brinker, A. M.; Powell, R. G. Glucosinolate composition of seeds from 297 species of wild plants. *Phytochemistry* **1991**, *30*, 2623–2638.

Fenwick, G. R.; Heaney, R. K.; Mullin, W. J. Glucosinolates and their breakdown products in food and food plants. *CRC Crit. Rev. Food Sci. Nutr.* **1983**, *18*, 123–201.

Goodman, I.; Fouts, J. R.; Bresnick, E.; Menegas, R.; Hitchings, G. H. A mammalian thioglycosidase. *Science* **1959**, *130*, 450–451.

Guo, Z.; Smith, T. J.; Wang, E.; Sadrieh, N.; Ma, Q.; Thomas, P. E.; Yang, C. S. Effects of phenethyl isothiocyanate, a carcinogenesis inhibitor, on xenobiotic-metabolizing enzymes and nitrosamine metabolism in rats. *Carcinogenesis* **1992**, *13*, 2205–2210.

Hecht, S. S. Chemoprevention by isothiocyanates. *J. Cell. Biochem.* **1995**, *22* (Suppl.), 195–209.

Hecht, S. S.; Chung, F-L.; Richie, J.P., Jr.; Akerkar, S. A.; Borukhova, A.; Skowronski, L.; Carmella, S. G. Effects of watercress consumption on metabolism of a tobacco-specific lung carcinogen in smokers. *Cancer Epidemiol. Biomarkers Prev.* **1995**, *4:* 877–884.

Hoglund, A. S.; Lenman, M.; Falk, A.; Rask, L. Distribution of myrosinase in rapeseed tissues. *Plant Physiol.* **1991**, *95*, 213–221.

Jiao, D.; Ho, C-T.; Foiles, P.; Chung, F-L. Identification and quantitation of *N*-acetylcysteine conjugate of allyl isothiocyanate in human urine after ingestion of mustard. *Cancer Epidemiol. Biomarkers Prev.* **1994**, *3*, 487–492.

MacLeod, A. J.; Panesar, S.; Gil, V. Thermal degradation of glucosinolates. *Phytochemistry* **1981**, *5*, 977–980.

Mennicke, W. H.; Görler, K.; Krumbiegel, G.; Lorenz, D.; Rittmann, N. Studies on the metabolism and excretion of benzyl isothiocyanate in man. *Xenobiotica* **1988**, *18*, 441–447.

Negri, E.; La Vecchia, C.; Franceschi, S.; D'Avanzo, B.; Parazzini, F. Vegetable and fruit consumption and cancer risk. *Int. J. Cancer* **1990**, *48*, 350–354.

Nijhoff, W. A.; Mulder, T. P. J.; Verhagen, H.; van Poppel, G.; Peters, W. H. M. Effects of consumption of brussels sprouts on plasma and urinary glutathione S-transferase class-α and -π in humans. *Carcinogenesis* **1995**, *16*, 955–957.

Nishie, K.; Daxenbichler, M. E. Toxicology of glucosinolates, related compounds (nitriles, R-goitrin, isothiocyanates) and vitamin U found in cruciferae. *Food Cosmet. Toxicol.* **1980**, *18*, 1191–1194.

Oginsky, E. L.; Stein, A. E.; Greer, M. A. Myrosinase activity in bacteria as demonstrated by the conversion of progoitrin to goitrin. *Proc. Soc. Exp. Biol. Med.* **1965**, *119*, 360–364.

Rodgers, A. E.; Zeisel, S. H.; Groopman, J. Diet and carcinogenesis. *Carcinogenesis* **1993**, *14*, 2205–2217.

Snedecor, G. W.; Cochran, W. G. *Statistical Methods*, 6th ed.; The Iowa State University Press: Ames, IA, 1967.

Sparnins, V. L.; Venegas, P. L.; Wattenberg, L. W. Glutathione S-transferase activity: enhancement by compounds inhibiting chemical carcinogenesis and by dietary constituents. *J. Natl. Cancer Inst.* **1982**, *68*, 493–496.

Steinmetz, K. A.; Potter, J. D. Vegetables, fruit, and cancer. I. Epidemiology. *Cancer Causes Control* **1991**, *2*, 325–357.

VanEtten, C. H.; Tookey, H. L. Glucosinolates in cruciferous plants. In *Herbivores, Their Interaction in Secondary Plant Metabolites*; Rosenthal, G. A., Janzen, D. H., Eds.; Academic Press: New York, 1979; pp 507–520.

Willett, W. C. Micronutrients and cancer risk. *Am. J. Clin. Nutr.* **1994**, 1162s–1165s.

Zhang, Y.; Cho, C.; Posner, G. H.; Talalay, P. Spectroscopic quantitation of organic isothiocyanates by cyclocondensation with vicinal dithiols. *Anal. Biochem.* **1992**, *205*, 100–107.

Zhang, Y.; Wade, K. L.; Prestera, T.; Talalay, P. Quantitative determination of isothiocyanates, dithiocarbonates, carbon disulfide, and related thiocarbonyl compounds by cyclocondensation with 1,2-benzenedithiol. *Anal. Biochem.* **1996**, *239*, 160–167.