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Full Length Research Paper

Development and verification of sulforaphane extraction method in cabbage (*Brassica oleracea* L. var*. capitata*) and broccoli (*Brassica oleracea* L. var*. italica* Planch.)

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A simple and repeatable method of extracting sulforaphane from cabbage and broccoli was developed and two inbred lines of broccoli with higher sulforaphane content in their seeds were found. The determination method was reverse-phase high performance liquid chromatography (RP-HPLC). In the extraction process, low toxic solvent ethyl acetate was applied for extraction of sulforaphane. The result showed that the linear equation was Y = 5907.07X + 4556.71, R^2 = 0.9996 assistant with HPLC conditions, and there was a stable and repeatable HPLC condition with the recovery of 96.3% (n = 6, relative standard deviation (RSD) = 1.3%). The better hydrolysis conditions for sulforaphane generation were at pH 7.0, the ratio of buffer to materials (ml/g) 5 to 20:1 and reaction time 1.0 to 2.0 h at room temperature. Finally, eighteen cultivars of cabbage and twelve cultivars of broccoli were used for verification of sulforaphane exaction method. All the materials were proved that they all contained sulforaphane composition and two cultivars of broccoli were proved to contain higher sulforaphane in seeds than previous reports.

Key words: Glucosinolate, sulforaphane, *Brassica*, broccoli, cabbage, reverse-phase high performance liquid chromatography (RP-HPLC).

INTRODUCTION

Cruciferous vegetables have been reported as rich sources of glucosinolate (Walters et al., 2004). When cruciferous vegetables are cut, chopped or chipped, myrosinase that exists in a specific protein body is released and mixes with glucosinolate, and myrosinase breaks the β-thioglucoside bond of glucosinolate molecules, producing glucose, sulfate, nitrile, thiocyanates and isothiocyanates. Sulforaphane (1-isothio-cyanato-4-methylsulfinylbutane, SF) is the hydrolysis product of glucoraphanin (4-methylsulfinybutyl glucosinolate, RAA) (Hakooz and Hamdan, 2007). Liang et al. (2006) reported that glucoraphanin can hydrolyze to sulforaphane at neutral condition, but provide no detailed experimental data. Epidemiological studies suggest that consumption of cruciferous vegetables, especially broccoli and brussels sprouts, can reduce the risk of cancers (Fawzy and Nehad, 2011), such as stomach (Hansson et al., 1993), lung (Spitz et al., 2000; Zhao et al., 2001), prostate (Canene et al., 2007; Kirsh et al., 2007), colon and rectum (Lin et al., 1998; Seow et al., 2002), breast (Fowke et al., 2003; Ambrosone et al., 2004) and bladder (Zhang, 2000; Zhao et al., 2007) and so on, as well as myocardial infarction (Cornelis et al., 2007). Many studies revealed that sulforaphane play a key role in causing cell cycle arrest and apoptosis (Thejass and Kuttan, 2007).

Methods for sulforaphane analysis have already been reported. These methods include gas chromatography (GC) (Spencer and Daxenbichler, 1980), GC with mass spectrum (GC-MS) (Chiang et al., 1998) and high performance liquid chromatography (HPLC) (Bertelli et al., 1998). However, sulforaphane will degrade at high temperatures (Liang et al., 2006). Furthermore, most

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Methods of determination are time-consuming, mainly wasting time on extraction process. At the same time, most of solvents reported are methanol, dichloromethane or acetone. These solvents are highly toxic or strong oxidant even though some of them are with a high recovery, but there are still some problems with them, because sulforaphane is unstable at strong oxidant conditions. So a simple and repeatable method of extracting sulforaphane is of valuable research; and, it is also important for the reduction of toxic residues in the extracts in a long time.

Most literatures about glucosinolates in *Arabidopsis thaliana* or in some *Brassica* plants have been reported (Fahey et al., 2001; Brown et al., 2003; Jones et al., 2006; Agerbirk and Olsen, 2012), however, most of the glucosinolates and their hydrolysis products provide no benefits to the human. The bioactivity components of glucosinolates are mainly glucoraphanin and glucobrassicin (hydrolysis product is indole-3-carbinol, I3C). Because *Brassica* vegetable especially such as broccoli and broccoli sprouts are usually reported rich in glucoraphanin. So many people mainly pay attention to the normal vegetables with higher content of sulforaphane, like broccoli and cabbage. Some seeds of Chinese *Brassica* species have been screened for determination of anti-cancer sulforaphane (You et al., 2008), but there are less materials of cabbage cultivars (nine) and broccoli (seven), and the highest content of sulforaphane is found in broccoli seed (1.58 g/kg) which content is further lower than 11.91 g/kg detected in our inbred line ZQII. So far, there are hardly reports higher than the content of sulforaphane in the study, and which is good for studies on purification and extract of sulforaphane from natural plant, as well as medical research. At the same time there is little report of sulforaphane content in fresh part of cabbage and broccoli combined based on so many cultivars (Koo et al., 2011).

In this paper, we reported an easy and repeatable method of extracting sulforaphane from cabbage and broccoli, and provided detailed parameters in extraction process. Moreover, reverse-phase HPLC (RP-HPLC) was used for determination of sulforaphane, which might be conducive to the studies on medicine and anti-cancer drug as well as biochemistry. At the same time, because just broccoli seed have been reported to be with higher content of sulforaphane, so five inbred lines of broccoli with stable agronomic traits were collected to screen their seed for the component of sulforaphane, the other materials were collected to understand the sulforaphane levels in the edible part and non-edible part of cabbage and broccoli.

MATERIALS AND METHODS

Standard and reagents

Sulforaphane standard was purchased from LKT Biochemical Company™ (USA), and the purity was above 99.0%. Methanol was HPLC grade and purchased from Sigma-Aldrich™ (USA). Ultra-pure water was made by Milli-Q quality water system (Millipore Bedford™, USA). Chemical K_2HPO_4, KH_2PO_4 and ethyl acetate of analysis reagents were purchased from Beijing chemical works™ (CHINA).

Plant materials

Twelve cultivars of broccoli and eighteen cultivars of cabbage were planted in field in the autumn 2009. All the materials were set three repeats and line spacing was 30 × 40 cm. When they were in commodity period, edible parts of cabbage and broccoli resources were collected; in addition, leaves and edible stems being 20 to 30 cm from florets to root of broccoli plants were individually collected. At the same time, five typical lines of broccoli with stable and good agronomic traits named ZQI (inbred line), ZQII (inbred line), ZQIII (inbred line), GRI (F_1) and GRII (F_1) were also collected for analysis of sulforaphane content in their seeds. And all the seeds were obtained from Chinese Institute of Vegetables and Flowers (IVF), Chinese Academy of Agricultural Sciences (CAAS).

Sample preparation and extraction process

The gathering samples were all dried by vacuum freeze-drying machine, and then dry samples were crushed into powder mechanically. And 0.50 g sample powder was accurately weighed and homogenized with 10.0 ml phosphate buffer (K_2HPO_4 and KH_2PO_4, 0.1 mol, pH 7.0), shaking for 1 h in a flask, and then it was extracted by 30 ml ethyl acetate. All the solutions were transferred to 50 ml tube, and then it was centrifuged for 10 min at 6, 000 × *g*. The supernatant was collected and the residual liquid was extracted by ethyl acetate until the third time using the same process. And then all collected supernatant was evaporated by rotavapor (RII-BÜCHI™, Switzerland) at 35°C in water bath under reduced pressure. The dry residue was then dissolved in 10 ml methanol, being filtered through Angela™ (USA) No. 0.22 μm nylon filter (D 13 mm) and stored at -20°C for determination.

In the study, different pH values, reaction time (hour) and the ratio of buffer to materials (g/ml) were set for choosing detailed parameters of extraction by the seed of GRII. They are individually as follows: pH values 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0; reaction times (hour) 0.5, 1.0, 2.0, 3.0, 4.0, 5.0 and 6.0; the ratios of buffer to materials (ml/g) 2.5, 5.0, 10.0, 20.0, 30.0, 40.0 and 50.0.

HPLC conditions

A SHIMADZU LC-20A HPLC system equipped with a SPD-20 ultraviolet (UV) detector and a reverse-phase C_{18} column (250 × 4.6 mm, 5 μm, SHISEIDOTM ™) were used for determination of sulforaphane.

A gradient mobile phase, which consisted 5% tetrahydrofuran (A) solution and 100% methanol (B), was chosen. The programme followed that B was 40% initially and then changed linearly to 60% in 10th min, and return to 100% after 10th min, equilibrating for 15 min at a flow rate of 0.80 ml/min. The absorbance was at 254 nm, and oven temperature was set at 32°C. The injection volume was 10 μl.

External standard method was used for the determination of sulforaphane, and 10.0 mg standard sample of sulforaphane was dissolved in 10 ml methanol, and then it was diluted to different concentrations of standard solution ranged from 0.5 to 100.0 mg/l.

The peak areas of one standard and one sample were recorded, and precisions of the system were calculated by the relative

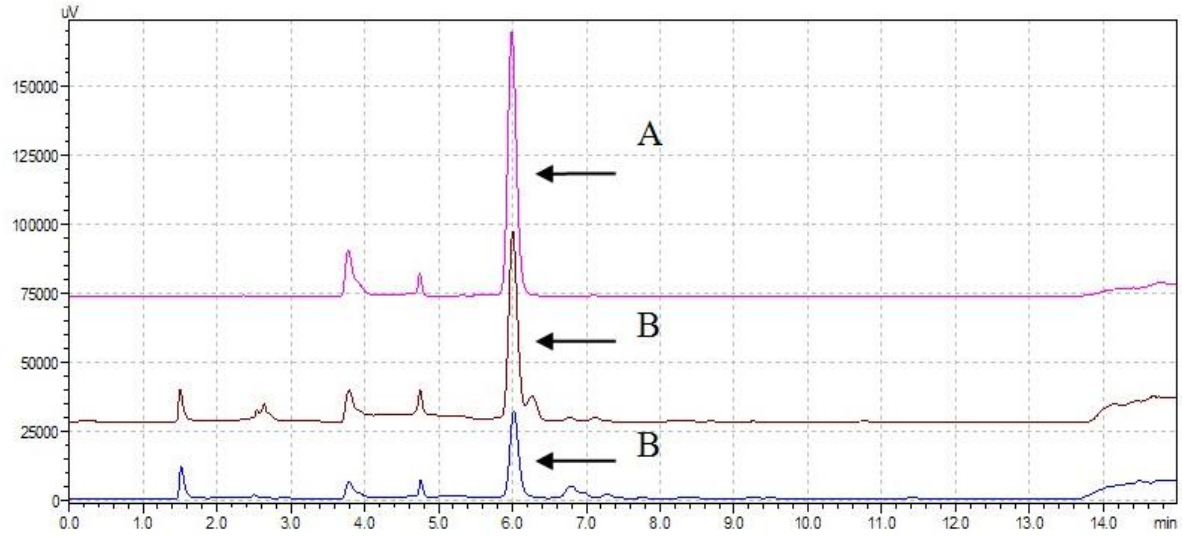

Figure 1. Chromatograms of standard (A) and sample extracts (B).

standard deviation (RSD) of the peak areas (n = 6). Recovery was analyzed by spiking known amount of sulforaphane in samples (n =6). In the study, we detected one same standard by keeping the record every 2 h (n = 5).

Data analysis

Data was represented as average ± standard deviation (Mean ± SD) (n = 3), one-way analysis of variance (ANOVA) was analyzed by SPSS 10.0, and pictures was treated by Sigma plot 10.0.

RESULTS AND DISCUSSION

HPLC conditions

The peaks of sulforaphane were clearly and accurately identified in standard and samples, and the retention time was about 6.08 min (Figure 1). Data showed that the linear equation was based on the peak area responses (Y) and theoretical concentration (X): $Y = 5907.07X + 4556.71$, $R^2 = 0.9996$ (Figure 2).

The data showed that the RSD (%) of standard and extracted samples were $2.5e^{-9}$ and 0.81, respectively (n = 6). The average recovery for sulforaphane was 96.2 ± 1.3% (n = 6), which was higher than some reports about extraction percentage of sulforaphane (You et al., 2008), and the RSD of stability was 0.22% (n = 5). So, there was a stable and reliable HPLC system for determination of sulforaphane.

Reaction time

It suggested that there was higher content of sulforaphane after 1 or 2 h hydrolysis reaction (Figure 3) and the statistics data showed that there were no significant differences at 1%, but there were significant differences between both of them and the other treatments; and the highest content after 1 h hydrolysis was about 1.10 to 1.51-fold higher than the others, which was consistent with previous report about hydrolysis time good for sulforaphane generation (Liang et al., 2006).

Reaction time can affect the bioactivity of many enzymes, so there will be less yield of sulforaphane in a too long or too short time (Hashem et al., 2011; Yabar et al., 2011), the study provide an optimization time for higher yield of sulforaphane.

pH value

Studies show that there are different hydrolysis products of glucosinolates at different pH conditions. It is generally published that low pH with ferrous ion and epithiospecifier protein (ESP) are beneficial to formation of nitrile when glucosinolates are broken by myrosinase (Liang et al., 2006). On the other hand, the activity of myrosinase and the ratio of isothiocyanates to nitriles formed in the disrupted tissue of cruciferous vegetables (Liang et al., 2006).

In the study, we could clearly see that there were different contents of sulforaphane at different pH (Figure 4), and it might reveal that myrosinase might play a key role in catalyzing RAA hydrolyzed to sulforaphane at pH 7.0, because the data showed that the content of sulforaphane at pH 7.0 was about 1.07 to 1.19-fold higher than those at the other pH values. So, we could get higher hydrolysis product of sulforphane at neutral conditions, which also proved others report (Liang et al., 2006). At the same time the result was almost consistent with pH optima for myrosinase activity in crude extracts from cruciferous crops (Charron and Sams, 2005), which also suggested that multiple factors and not only one

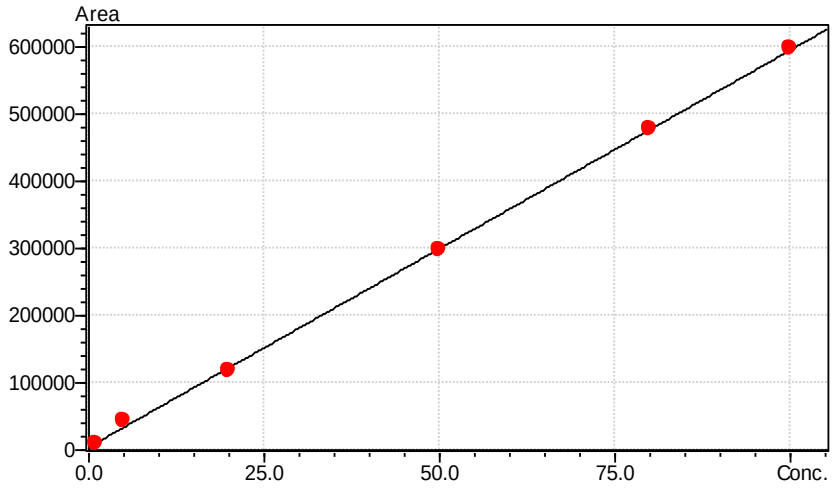

Figure 2. Standard curve of sulforaphane.

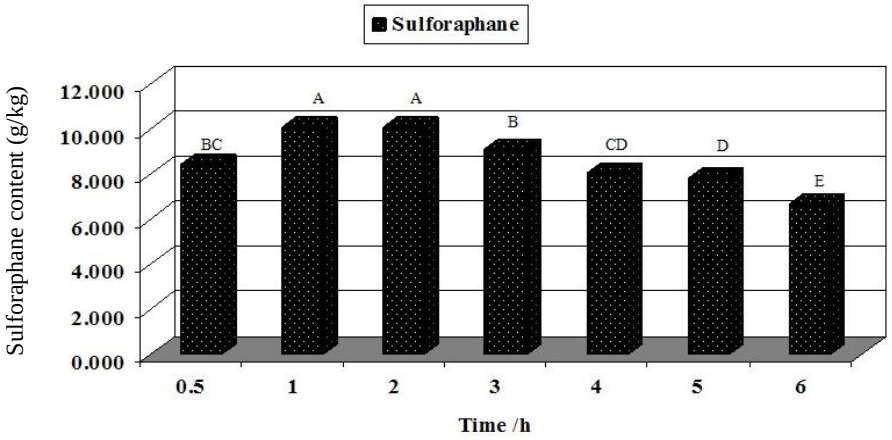

Figure 3. Contents of sulforaphane after different hydrolysis time, and different capital letters meant significant differences at 1%.

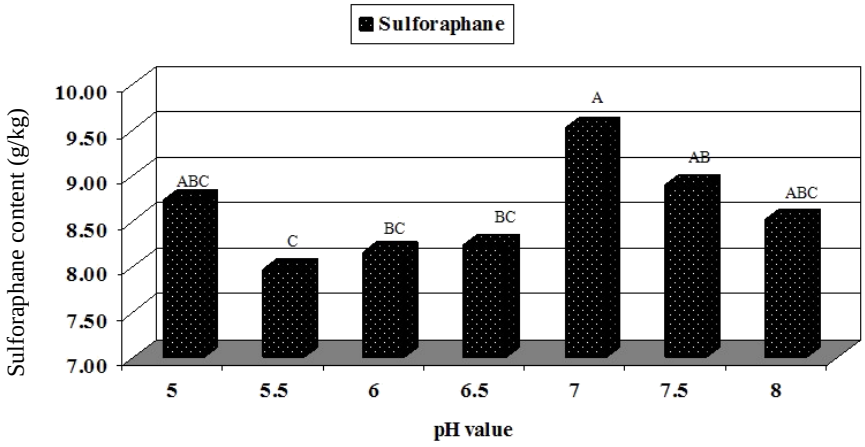

Figure 4. Contents of sulforaphane at different pH values and different capital letters meant significant differences at 1%.

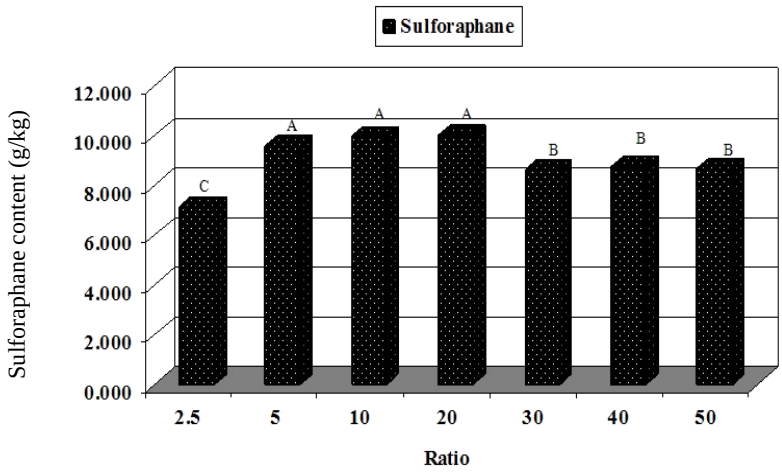

Figure 5. Contents of sulforaphane in different ratios of buffer to material (ml/g) and different capital letters meant significant differences at 1%.

Table 1. Sulforaphane content in the edible part of eighteen cultivars of cabbage[a].

Cultivar	Content (mg/kg) DW[b]	Line	Content (mg/kg) DW
G3	6.54 ± 1.73	G5	19.73 ± 4.78
G18	15.09 ± 3.01	G19	10.64 ± 2.96
G255	33.18 ± 5.29	G290	5.73 ± 0.46
G213	25.91 ± 4.73	G285	52.00 ± 12.88
G234	13.91 ± 3.03	G279	8.18 ± 2.51
G238	10.27 ± 2.32	G276	16.73 ± 4.04
G258	42.82 ± 6.99	G409	25.09 ± 5.06
G266	49.64 ± 10.22	G210	38.91 ± 7.24
G316	3.91 ± 1.27	G220	39.73 ± 8.86

[a], Values represent means ± SD (n = 3); [b], dry weight.

affected the yield of sulforaphane, and the same pH condition and conclusion have been reported in horseradish (Mucete et al., 2006).

The ratio of buffer to material

It showed that there were no significant differences in the ratios of buffer to material (ml/g) 5:1, 10:1 or 20:1 at 1% (Figure 5), and the highest content of sulforaphane in the ratio of 20:1 (A) was about 1.15 to 1.41-fold higher than the other conditions (B and C). So the ratios of 5 to 20:1 might be good for sulforaphane generation by providing a stable and good condition for myrosinase.

Sample verification

The data showed that all the materials were proved to contain sulforaphane compound (Tables 1 and 2). The contents of sulforahane in cabbage ranged from 3.91 to 52.00 mg/kg dry weight (DW), while they were 62.64 to 982.36 mg/kg DW in florets of broccoli; 18.11 to 274.00 mg/kg DW in stems of broccoli; 6.55 to 256.46 mg/kg DW in leaves of broccoli. The result showed a huge difference in the content of sulforaphane, some reports revealed that the content of sulforaphane are caused by genotype or interaction of genotype and environment (Kliebenstein et al., 2001; Li and Quiros, 2002; Canistro et al., 2004; Farnham et al., 2004; Wentzell et al., 2008). The similar result has been also reported in cabbage [0.7 to 5.3 mg/kg fresh weight (FW)] and the same three parts of broccoli (florets: 1.4 to 32.9 mg/kg FW; stalks: 0.8 to 9.3 mg/kg FW; leaves 0.3 to 4.3 mg/kg FW) (Liang et al., 2006).

So, it seemed that in all the materials the content of sulforaphane in cultivar B76 was higher than the others in the organ of florets, which also happened in the organ of stems and leaves of B86. The highest contents of sulforaphane in florets, stems and leaves were separately

Table 2. Sulforaphane content in different organs of twelve cultivars of broccoli[a].

Cultivar	Florets	Stems	Leaves
	Content (mg/kg) DW[b]	Content (mg/kg) DW	Content (mg/kg) DW
B47	354.82 ± 32.44	39.78 ± 4.01	6.55 ± 2.65
B56	252.18 ± 20.76	41.44 ± 4.86	36 .00 ± 4.98
B58	93.27 ± 10.62	33.67 ± 3.23	38.01 ± 4.27
B50	62.64 ± 5.98	64.33 ± 6.89	10.45 ± 3.33
B79	515.82 ± 48.48	157.67 ± 14.17	105.73 ± 12.46
B161	432.09 ± 40.55	96 .01± 11.03	106.45 ± 11.09
B20	310.91 ± 33.01	240.01 ± 20.01	97.91 ± 10.72
B86	412.09 ± 40.89	274.00 ± 29.22	256.46 ± 28.48
B604	165.45 ± 18.11	111.78 ± 12.99	54.82 ± 5.99
B167	77.18 ± 6.26	18.11 ± 4.76	44.91 ± 5.05
B76	982.36 ± 69.03	72.33 ± 8.9	80.27 ± 8.89
B91	562.91 ± 45.00	272.00± 31.88	74.27 ± 10.01
Mean	351.73	118.44	76.00

[a], Values represent means ± SD (n = 3); [b], dry weight.

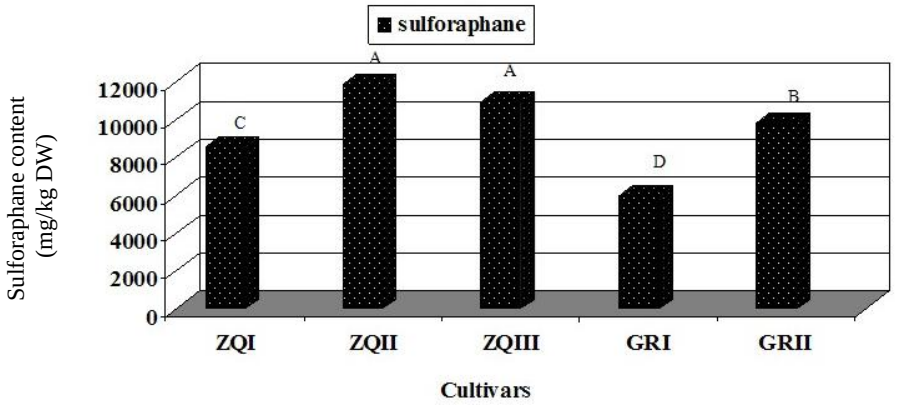

Figure 6. Sulforaphane contents in five cultivars' seed of broccoli and different capital letters meant significant differences at 1%.

15.7, 15.1 and 39.2-fold higher than the least level in relative organs. The result was consistent with previous data (Liang et al., 2006).

Five cultivars of broccoli with good agronomic traits (ZQI, ZQII, ZQIII, GRI and GRII) were all found to contain higher contents of sulforaphane in their seed, and there were significant differences at 1% (Figure 6). Matusheski et al. (2004) reported the highest sulforaphane content 4.80 g/kg DW in broccoli seed and stated that the content is of valuable industrial production of sulforaphane. Liang et al. (2004) also reported the detection result in seeds of broccoli, in which the highest content of sulforaphane is about 4.62 g/kg DW. In the study, five cultivars' seeds of broccoli were found that their average sulforaphane contents (n = 3) were individually 8.56, 11.91, 10.93, 5.98 and 9.82 g/kg DW (ZQI, ZQII, ZQIII, GRI and GRII), and all of them were higher than some previous reports; inbred lines of ZQII and ZQIII might be good natural resources for sulforaphane extraction and breeding of vegetables. In other way, it could be inferred from the result that variation of sulforapahane contents might be mainly affected by genotype according to the data from different cultivars and two species, which was also consistent with previous research (Abercrombie et al., 2005; Gasper et al., 2011). So excavation of good natural with higher sulforaphane content in broccoli may be conducive to the study of the anti-cancer drug and medicine research.

In the study, we have also developed a simple and repeatable extraction method of sulforphane based on ethyl acetate as low toxic solvent. The innovative work in the study were that different gradient conditions in pH,

reaction time and the ratio of buffer to the materials were investigated to obtain a optimization hydrolysis condition. At the same time, lower toxic solvent ethyl acetate was applied for extraction of sulforaphane based on a higher recovery system (Agrawal et al., 2006; Wang et al., 2011), and finally two inbred lines of broccoli was found to be with higher sulforaphane contents in their seeds than previous levels. The better hydrolysis conditions were following: 1 or 2 h hydrolysis reaction, pH 7.0 and at the ratio of buffer to material (ml/g) 5 to 20:1 at room temperature.

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