III EXPERIMENTAL

3.1 Apparatus and materials

3.1.1 Apparatus

Standard laboratory apparatus have been employed a long with, in particular, the following:

1 High-performance liquid chromatography apparatus – Hewlett Packard model 1090 series II/L system equipped with a quaternary pump 79835A, a UV-Vis photodiode array detector 79883A, with an automatic sample injector 79846A, a column oven 79847A and an on-line vacuum degasser G1303A. This instrument was installed with HP ChemStation data software version 6.03 (Agilent Technologies, Waldbronn, Germany).

2 Analytical balance – Mettle Toledo, model AB 2004-S, Switzerland, capable of weighing 0.0001-220 g.

3 Autosampler vials and caps amber – Sun, # 200-406, USA.

4 Centrifuge – Fischer Scientific, model 1987B with cooling system, max. 11,000 rpm, with Nalgene 85 mL Teflon FEP tubes, Switzerland.

5 Furnace – Sybron Thermolyne, model F6020, # 328C0289, USA.

6 Micropipette – Pipettman, L-20 (2-20 μL), L-100 (10-100 mL) and L-1000 (100-1000 μL), USA.

7 Orbital shaker – Merck, model IKA Werk KS500 (50-600/min), Switzerland.

8 pH meter – Precisa pH 900, Swissmade, Switzerland.

9 Rotary evaporator – Eyela, model N-1000SWB, Japan.

10 Shaking machine – Shaking Water Bath, model SB-200-10, Thailand.

11 Spectrophotometer – Genesys 2, USA.

12 Ultrasonic bath – Elma, T710DH, Germany.

3.1.2 Broccoli seeds

The research focused specifically in various broccoli seeds cultivars available in Thailand under commonly-growth conditions in Thailand. There were five broccoli seeds cultivars which included 'Green Queen' (กรีนควีน), 'Packman' (แพคแมน), 'Pak Ging' (ปักกิ่ง), 'Rod Fai' (รถไฟ), and 'Top Green #067' (ท็อปกรีน #067). Label claims of broccoli seeds in each cultivar were shown in appendices A1-A5, page 131-133. During the 2002 winter season, broccoli seeds were sown directly in seed-bed container. Young plants were planted out when they reached the five-to seven-true leaf stage into the field. When environmental conditions in the field was expected to remain favourable for further flower development until the seeds can be harvasted. This period lasted approximately 60-150 days depending on the type of broccoli cultivar (appendix B, page 134). These seeds were obtained from Phu Ruea Highland Cultivation Experimental Station site, Loei province, Thailand. All seeds were harvested in April-May, 2003 and were packaged and sealed in aluminium foil laminated sachets. The seeds were storaged, unopened and refrigerated in 4° C condition until use.

3.1.3 Chemicals and reagents

All chemicals and reagents were analytical grade unless otherwise specified.

1 ABTS [2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)] diammonium salt, Sigma-Aldrich Chemical Co., St. Louis, USA.

2 Acetonitrile, HPLC grade, Fisher, Italy.

3 Acetic acid (glacial), J.T.Baker, USA.

4 Aluminium trichloride hydrate, Labscan Asia, Thailand.

5 L-ascorbic acid, Fisher, UK.

6 β-Carotene, Sigma Chemical Co., USA.

7 (+)-Catechin, Sigma Chemical Co., USA.

8 Certified rapeseed reference materials BCR-367R, Commission of the European Community Bureau of References, Brussels, Belgium.

9 Chloroform, HPLC-grade, Merck, Germany.

10 DEAE Sephadex A25, Sigma Chemical Co., USA.

11 (-)-Epicatechin, Sigma Chemical Co., USA.

12 (-)-Epigallocatechin gallate, Sigma Chemical Co., USA.

13 Ethanol, HPLC grade, Fisher, Italy.

14 Ethanol, Merck, Germany.

15 Ether, Merck, Germany.

16 Ethyl acetate, Merck, Germany.

17 Folin-Ciocalteu phenol reagent, 2.0 M, Sigma Chemical Co., USA.

18 Formic acid, Merck, Germany.

19 Gallic acid, Sigma Chemical Co., USA.

Glucosinolates, individual standards, Sigma Chemical Co., St. Louis, USA.

n-Hexane, Labscan Asia, Thailand.

Hydrocholric acid, Merck, Germany.

Iron (III) chloride heptahydrate, BDH, England.

Imidazole, Merck, Germany.

Methanol, HPLC grade, Fisher, Italy.

Methanol, Merck, Germany.

Petroleum ether, Merck, Germany.

m-Phosphoric acid, HPLC-grade, Merck, Germany.

Potassium hydroxide, Merck, Germany.

Potassium persulfate, Carlo Erba reagenti, Italy.

Quercetin, Sigma Chemical Co., USA.

Rutin (hydrate, min 95%), Sigma Chemical Co., USA.

Sodium acetate, Labscan Asia, Thailand.

Sodium carbonate (anhydrous), Sigma Chemical Co., USA.

Sulfatase (*Helix pomatia*) type H1 (EC. 3.1.6.1), Sigma Chemical Co., (catalog no. S9626), USA.

α-Tocopherol, Sigma Chemical Co., USA.

TPTZ [2,4,6-Tris(2-pyridyl)-s-triazine], Sigma Chemical Co, USA.

Trolox (6-hydroxy-2,5,7,8-tetramethylchlorman-2-carboxylic acid), Fluka, Switzerland.

Water, HPLC grade, was purified using a Milli-Q system from Millipore, Bedford, MA, USA.

3.1.4 Preparation of mobile phase

Prior to any HPLC analysis operation, all solutions were filtered through 0.45 μm membrane filter (Sartorius, Germany) and then degassed in an ultrasonic bath for 30 min.

3.2 General analytical methods

3.2.1 Moisture[124]

The hot air oven was seted and regulated to 135 ± 2° C. The broccoli seeds were weighed approximately 1.5-2 g into aluminium dish along with its covered lid, and then spread the seeds until the content was evenly distributed. With the covered lid was removed, the dish and the lid were placed next to each other in an oven as quickly as possible and dried for 2 h ± 5 min. Placed the lid to cover the dish and transferred to a dessicator for cooling. The dish was reweighed and calculated the loss in weight on drying (LOD) as determined from the amount of water loss.

$$\%(w/w)\ LOD = \%(w/w)\ moisture = \frac{100 \times \text{weight loss on drying, g}}{\text{weight test portion, g}}$$

$$\%\ Dry\ matter = 100 - \%LOD$$

3.2.2 Ash[125]

The broccoli seeds were weighed accurately about 2 g into a porcelain crucible and placed in a temperature controlled furnace which preheated to 600° C. The furnace

temperature was hold for 2 h. The crucible was transferred directly to a dessicator, cooled, and weighed.

$$\%(w/w)\ ash = \frac{100 \times (\text{weight of test portion, g} - \text{weight loss on ashing, g})}{\text{weight of test portion, g}}$$

3.2.3 Crude fat [126]

The ground broccoli seeds were accurately weighed about 2 g in to fat-extraction tube, 2 mL of 95% ethyl alcohol was added to prevent bumping on addition of acid and shaken to moisten all particles. Then 10 mL of 1 M HCl was added, mixed well, and set the tube for 30-40 min in a waterbath at 70-80° C, with shaking the tube frequently. The mixture was cooled to room temperature and added 25 mL of petroleum ether. The tube was closed with stopper and shaked vigorously for 1 min and carefully released the built up pressure so that no solvent was lost. The adhering solvent and fat were washed from stopper back into the extraction tube with a few mL of petroleum ether. The 25 mL petroleum ether was added and shaken vigorously for 1 min. The solution was then allowed to stand until separation occurred and the upper liquid layer is practically clear. The ether-fat solution was poured through filter consisting of cotton pledget packed in the funnel stem into 150 mL beaker. The fat tube was re-extracted twice, each time with only 15 mL of ether, shaking 1 min after addition of ether. The solvents were combined and evaporated, slowly on steam bath, then cooled to room temperature.

The dried fat residue was redissolved in four 10 mL portions ethyl ether, then filtering each portion through small pledget of cotton into 100 mL beaker that had

been predried for 30 min at 100° C, cooled to room temperature in a dissiccator, and weigh immediately. The fifth 10 mL portion of ether was used for rinsing the cotton pledget and funnel. The ether was evaporated on steam bath, dried 90 min at 100° C, cooled to room temperature in dissiccator, and weigh immediately.

3.3 Glucosinolates analysis

3.3.1 Extraction of glucosinolates from broccoli seeds

Broccoli seeds were ground with a mortar and pestle. A 0.2 g of ground sample from each replicate was placed in a capped 15 mL glass tube and heated on a heating block which set at 75° C for 1 min. Boiling methanol (2 mL) was then added to the test tube followed immediately by 0.25 mL of benzylglucosinolate (1 mg/mL) as an internal standard. The tube was continuously heated for 10 min and centrifuged at 3000 rpm for 3 min. After centrifugation, the supernatant was transferred to a 10 mL flask. The residue was extracted twice with 2 mL of boiling methanol and the solution was centrifuged each time. The supernatant was collected, combined with the previously saved supernatant, and mixed thoroughly in the same flask. Certified rapeseed reference material was performed the same procedure as described above.

3.3.2 Preparation of desulfoglucosinolates

3.3.2.1 Preparation of ion exchange resin

For suspension preparation, 10 g of DEAE Sephadex A25 resin was mixed in an excess 2 M acetic acid solution, allowed to completely swell and settle. Then, 2 M acetic acid was added until the volume of the liquid is equal to twice the volume of the sediment.

3.3.2.2 Praparation of ion exchange column

Six pasteur pipets (150 mm long) were required for five broccoli seeds extract cultivar and one for the certified rapeseed reference material. The pipets were placed with a glass wool pluged in the neck of each pipets and then placed vertically on the stand. The well-mixed suspension of ion-exchange resin (0.5 mL) were transferred to each pasteur pipet and allowed the resin to settle. The pipets were rinsed with 2 mL of 6 M imidazole formate (prepared by dissolving 204 g of imidazole in 113 mL of formic acid in a 500 mL volumetric flask and diluting to the mark with water), and then rinsed twice with 1 mL portion of water.

3.3.2.3 Preparation of purified sulfatase

Five pasteur pipets (7 cm) were inserted with glass wool plug in the neck and then placed vertically on the stand. 1 mL of 6 M imidazole formate solution was delivered into each pipet, and rinsed twice with 1 mL portion of water. Approximately 25 mg of sulfatase was weighed and dissolved in 2.5 mL of water.

Exactly 500 μL of this solution was transferred to each of the columns prepared above. The columns were washed with 1.5 mL of water each and discarded the effluent. Then 1.5 mL of a 0.2 M sodium acetate solution was added and collected the eluates from each of the columns into a labeled test tube. Concentrated the eluates by filtration using Millipore PTGC 11K252 immersion filter (Millipore, USA), until approximately 100 μL of liquid remains. Water (2.5 mL) was added and concentrated once more by filtration until approximately 100 μL of liquid remains. Each filtrate was diluted with 2.5 mL of water. This purified sulfatase solution, 1 mL, was transferred to a 10 mL volumetric flask, diluted to the mark with water and mixed well.

3.3.2.4 Preparation of desulfoglucosinolates

A 1 mL fraction of the supernatant from 3.3.1 section was transferred to DEAE Sephadex A-25 column from 3.3.2.2 section without disturbing the resin surface and allowed to drain. Two 1 mL portions of 0.02 M sodium acetate buffer, pH 4 were then added to the column, allowing the buffer to drain after each addition. Desulfoglucosinolates were obtained after this treatment of the column with purified sulfatase solution (500 μL) from 3.3.2.3 section. The column was capped for 12 h. Desulfated glucosinolates were eluted off from this column with three portions of 1 mL water and followed by final separation with HPLC.

3.3.3 Glucosinolates analysis by RP-PIC

The extracts from broccoli seed cultivars and the rapeseed reference materials were directly injected onto an analytical Lichrospher (150 x 2.1 mm i.d., 5 μm) RP-18 column, (Merck, Darmstat, Germany) with a mobile phase of acetonitrile (50%) plus de-ionized water (50%) containing 5 mM tetraoctylammonium bromide (solvent A) and acetonitrile (solvent B). The flow rate was 0.20 mL/min. Absorbance was monitored at 229 nm wavelength. The following gradient elution was used for separation: 0-20 min; 0%B, 20-23 min; 100%B, 23-30 min; 0%B. The column was extensively equilibrated with a minimum of 100 column void volumes of solvents. Injection volumn was 20 μL. At the end of each working day, the whole chromatographic system was rinsed with water-methanol (50:50 v/v) for 30 min. There was a 10 min post run wash out with the initial conditions for equilibration of the column. Data were processed with HP ChemStation software. Individual glucosinolate was identified by matching the retention times with those of known reference compounds and by adding separated, individual, pure compounds to broccoli seeds extracts and observing the raise in peak height. These techniques ensure the validity of the analytical procedure.

3.3.4 Method validation

For the validation of the analytical method: the AOAC guidelines for collaborative study procedures to validate characteristics of a method of analysis,[127] the guidelines for industry on analytical procedures and methods validation,[128] the guidelines of the International Conference on Harmonization (ICH) Guidelines[129] and US Pharmacopeia 26[130] were followed. Typical validation characteristics which are considered for the intended use of the method were listed below:

Accuracy

Precision

Specificity

Detection Limit (LOD)

Quantitation Limit (LOQ)

Linearity

Range

Robustness

System suitability testing

3.3.4.1 Accuracy

The accuracy of the method was evaluated with the recovery test. This involved an addition of known quantities of glucosinolates standards. The fortified samples were then extracted and analyzed with the proposed HPLC method. The percentage recovery was determined by subtracting the values obtained for the

control matrix preparation from those samples that were prepared with the added standards, divided by the amount added and then multiplied by 100.

3.3.4.2 Precision of the chromatographic system

Intra- and inter-day precision was tested by performing multiple injections of all the extracted broccoli seeds in each cultivar and then checking the percent relative standard deviation (%R.S.D.) of the retention times and peak areas. Ten injections were performed each day and the experiment was repeated for 3 consecutive days.

3.3.4.3 Precision of the extraction procedure

The precision of the extraction procedure was validated using certified rapeseed reference standard material. Five broccoli seed cultivars were extracted as described in section above. An aliquot of each extract was then injected and quantified.

3.3.4.4 Specificity

Peaks were identified on the basis of their retention time values and UV spectra by comparison with those of known glucosinolates compounds in the standard rapeseed reference materials. Peak identity was also confirmed by spiking the extracts with pure standards. Peak purity test was performed using a photodiode array detector coupled to the HPLC system, comparing the UV spectra of each peak with those of authentic reference standard.

3.3.4.5 Detection limit (LOD)

The detection limit of an individual anlaytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. Base on signal-to-noise, this approach was applied to the analytical procedures which exhibit baseline noise. Determination of the signal-to-noise was performed by comparing measured signals with known low concentrations of analyte with those of blank and establishing the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio of 3:1 is generally considered acceptable for estimimating the detection limit.

3.3.4.6 Quantitation limit (LOQ)

The quantitation limit of an individual anlaytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. Determination of the signal-to-noise was performed by comparing measured signals with known low concentrations of analyte with those of blank and establishing the minimum concentration at which the analyte can be reliably detected. A typical signal-to-noise ratio for the quantitation limit is 10:1.

3.3.4.7 Linearity

A linear relationship was evaluated across the range (*see* section 3.3.4.8) of the analytical procedure. Linearity should be evaluated by visual inspection of a plot of signals as a function of analyte concentration or content. If there is a linear

relationship, test results should be evaluated by appropriate statistical methods, for example, by calculation of a regression line or by the method of least squares. In some cases, to obtain linearity between assays and sample concentrations, the tested data may have to be subjected to a mathematical transformation prior to the regression analysis.

Data from the regression line itself may be helpful to provide mathematical estimation of the degree of linearity. The correlation coefficient, y-intercept, slope of the regression line, and residual sum of squares should be submitted. A plot of the data should be included. In addition, an analysis of the deviation of the actual data points from the regression line may also be helpful for evaluating linearity.

3.3.4.8 Range

The specified range is normally derived from linearity studies and depends on the intended application of the procedure. It is established by confirming that the analytical procedure provides an acceptable degree of linearity, accuracy, and precision when applied to samples containing amounts of analyte within or at the extremes of the specified range of the analytical procedure.

For this study, the linerity plot was constructed for analytical response versus 4-methylsulfinylbutyl glucosinolate standard concentration in rage 0.100-500 mg/L at the optimum condition which would had negligible intercept.

3.3.4.9 Robustness

Robustness, a measure of the analytical procedure's capability to remain unaffected by small but deliberate variations, is described in ICH *Q2A* and *Q2B*. The evaluation of robustness were considered during the development of the analytical procedure. It showed the reliability of an analysis with respect to deliberate variations in method parameters.

3.3.4.10 System suitability testing

System suitability testing parameters and acceptance criteria are based on the concept that the equipment, electronics, analytical operations, and samples to be analyzed constitute as an integrated system. System suitability testing ensures that the system is working properly at the time of analysis. Appropriate system suitability criteria were defined and included in this analytical procedure.

3.3.5 Calculation

3.3.5.1 The content of glucosinolates

The content of each glucosinolate, expresssed in micromoles per gram of dry matter of the product, is equl to

$$\frac{A_g}{A_s} \times \frac{n}{w_t} \times \frac{K_s}{K_g} \times \frac{100}{100-w_v} \quad (1)$$

Where: A_g – the peak area, in integrator units, corresponding to desulfoglucosinolate;

A_s – the peak area, in integrator units, corresponding to the internal standard used;

n – the quantity, in micromoles, of internal standard added to the tube;

w_t – the mass, in grams, of the test portion;

K_s – the response factor of the internal standard used;

K_g– the response factor of desulfoglucosinolate and

w_v – the moisture content, expressed as a percentage by weight, of the test sample.

3.4 Antioxidant assay

3.4.1 Total antioxidant capacity

The improved ABTS radical cation decolourization assay[131] and Ferric reducing antioxidant power (FRAP) assay[132] were used to systematically assess the total antioxidant capacity of broccoli seeds.

3.4.1.1 Preparation of sample

Broccoli seeds were milled and in turn extracted with five solvents: chloroform, ethyl acetate, ethanol (70%), methanol and water. Two step extraction was applied by shaking the flasks with 10 g (± 0.01 g) of seed and 100 mL (50 mL +50 mL) of solvent in a shaking machine. Each extraction step was completed in 3 h. The extracts were filtered, concentrated in a rotavapor evaporating apparatus at approximately 40 °C and then tested for the antioxidant activities.

3.4.1.2 ABTS radical cation decolourisation assay [131]

ABTS [2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)] radical cation decolourisation test is also a spectrophotometric method widely used for the assessment of antioxidant activity of various substances. ABTS was generated by oxidation of ABTS with potassium persulfate. Three mL of $ABTS^{\bullet+}$ cation solution was mixed with 30 mL seed extract solutions. A portion of this solution was transferred in 1 cm path length disposable microcuvette. The absorbance at 734 nm

was utilized then detected for antioxidant property after the solution was sit for the completion of the reaction for 4 min. The lower the absorbance represents the stronger the reducing power in comparison by using three known antioxidants. These substances included ascorbic acid as a vitamin C widely spread in fruits and vegetables, quercertin representing the group of polyphenol and Trolox as water soluble vitamin E analogue. The percentage of ABTS scavenging activity is expressed by [1-(test sample absorbance/ blank sample absorbance)] x 100.

3.4.1.3 Ferric reducing antioxidant power (FRAP assay)[132]

The FRAP assay as described by Benzie and Strain (1996) was used with minor modification. A 50 mL seeds extraction solution, combined with 30 mL water was pipetted and emptied in a 1 cm path length disposable microcuvette and use as blank. A 200 mL FRAP solution (300 mM acetate buffer pH 3.6: 10 mM TPTZ in 40 mM HCl: 20 mM $FeCl_3$; 10:1:1) was added to the seed extracted solution, mixed for 10s and took the measurement of absorbancy at 593 nm was taken after allowed 4 min for the reaction to complete. The higher the ferrous ion chelating ability of the test sample gives the lower absorbancy. The percentage of ferrous ion chelating ability is expressed by [1-(test sample absorbance/ blank sample absorbance)] x 100.

3.4.2 Total Phenolics, flavonoids and flavonols compounds assay

3.4.2.1 Preparation of sample

Broccoli seeds were ground with a mortar and pestle and then extracted with methanol (99.5%). Two-step extraction was applied by the shaking flasks with 10 g (± 0.01 g) of seeds and 100 mL (50 mL + 50 mL) of solvent in a shaking machine. Each extraction step was completed within 3 h. The extracts were filtered, concentrated in a rotary evaporator apparatus at approximately 40° C. A spectrophotometer was used for total phenolics, flavonoids and flavonols compounds measurement.

3.4.2.2 Total phenolics assay

The total content of phenolic compounds in broccoli seeds methanolic extracts was determined by Folin–Ciocalteu method.[133] For the preparation of calibration curve, 1 mL aliquots of 0.024, 0.075, 0.105 and 0.3 mg/mL ethanolic gallic acid solutions were mixed with 5 mL Folin–Ciocalteu reagent (diluted ten-fold) and 4 mL (75 g/L) sodium carbonate. The absorption was read after 30 min at 20° C at 765 nm and the calibration curve was drawn. One mL methanolic seed extract was mixed with the same reagents as described above, and after 1 h the absorption was measured for the determination of seed phenolics. All determinations were performed in triplicate. Total content of phenolic compounds in seed methanol extracts in gallic acid equivalents (GAE) was calculated by the following formula:

$$C = c\,(V/m') \qquad (2)$$

where: *C*–total content of phenolic compounds, mg/g seed extract, in GAE;

c–the concentration of gallic acid established from the calibration curve, mg/mL;

V–the volume of extract, mL;

m'–the weight of pure seed methanolic extract, g.

3.4.2.3 Flavonoids and flavonols assay

The content of flavonoids was determined by a USP method, 1989[134] using rutin as a reference standard compound. One mL of broccoli seed extract in methanol was mixed with 1 mL aluminium trichloride in ethanol (20 g/L) and diluted with ethanol to 25 mL. The absorption at 415 nm was read after 40 min at 20° C. Blank samples were prepared from 1 mL of the seed extract and 1 drop of acetic acid was added, and diluted to 25 mL. The absorption of rutin solutions was measured under the same conditions. All determinations were also carried out in triplicate.

The content of flavonols was determined by the Yermakov method, 1987.[135] The rutin calibration curve was prepared by mixing 2 mL of 0.1, 0.2, 0.3, 0.4 and 0.5 mg/mL rutin in ethanolic solutions with 2 mL (20 g/L) aluminum trichloride and 6 mL (50 g/L) sodium acetate. The absorption at 440 nm was read after 2.5 h at 20° C. The same procedure was carried out with 1 mL of broccoli seeds extracts instead of rutin solution. All determinations were carried out in triplicate.

The amount of flavonoids and flavonols in seed extracts in rutin equivalents (RE) was calculated by the following formula:

$$X = C\,(V/m) \qquad (3)$$

where: *X*–flavonoids content, mg/g seed extract in RE;

C–the concentration of rutin solution, established from the calibration curve, mg/mL;

V, m–the volume and the weight of seed extract, mL, g.

3.4.3 Individual antioxidant activity

3.4.3.1 Phenolic compound analysis

A high-performance liquid chromatographic (HPLC) separation method with photo-diode array detection has been developed for the simultaneous determination of the main phenolics classes including, catechin, epicatechin, epigallocatechin gallate, gallic acid, quercetin and rutin.

3.4.3.1.1 Preparation of sample

Approximately 10 g of the seeds were ground with a pestle and a mortar, and then transferred to a centrifuge tube with 10 mL of aqueous methanol (80%, v/v) in diluted HCl acid media (1%, v/v). The mixture was centrifuged at 5000 rpm for 20 min and this process was repeated twice. The supernatant was filtered through a 0.45 μm Sartolon polyamide membrane filter (Sartorius, Germany) before injection.

3.4.3.1.2 Preparation of standard solution and working standard solution

The standard solutions containing about 50 ppm each of catechin, epicatechin, epigallocatechin gallate, gallic acid, quercetin and rutin were prepared in methanol and used in the method validation and analysis of broccoli seeds samples. All standard solutions were stored in dark place and kept at 5° C in a laboratory refrigerator.

3.4.3.1.3 HPLC analysis

The separation was achieved on a Waters Spherisorb 5 μm ODS (4.6 x 250 mm) column at 30° C. Columns were extensively equilibrated with a minimum of 100 column void volumes of solvents. The mobile phase consisted of acetonitrile (solvent A), acetic acid solution pH 3.0 (solvent B), and methanol (solvent C). The system was run with the following gradient elution program: 0 min, 5%A/95%B; 10 min, 10%A/80%B/10%C; 20 min, 20%A/60%B/20%C and 30 min, 100%A. There was a 10 min post run at initial condition for equilibration of the column. The flow rate was kept constant throughout the analysis at 1 mL/min. Injections were accomplished with a 10 μL fixed loop and the analysis was monitored at 280 nm. Identification was based on retention times and on-line spectral data in comparison with authentic standards. Quantification was performed by establishing calibration curves for each compound determined, using the reference standards.

3.4.3.2 Ascorbic acid extraction and assay

10 g of seeds were homogenized with 15 mL of 5% *m*-phosphoric acid. The homogenate was filtered with three layers of cheesecloth and the residue was treated with 10 mL of 5% *m*-phosphoric acid for two successive extractions. The filtrates were combined and centrifuged at 4000rpm for 10 min. The supernatant was collected and made up to 25 mL and then filtered through a 0.45 μm Sartolon polyamide membrane filter (Sartorius, Germany). The HPLC analytical column was 5 μm LiChrosorb RP-18 column, 250 x 4.6 mm (Merck, Darmstat, Germany). Ultra-pure water was generated by a Milli-Q system. The mobile phase was consisted of acidified distilled water (0.1% phosphoric acid) (solvent A) and acetonitrile (solvent B), at a ratio of 95:5 (A:B). The flow rate was set at 1.0 mL/min. L-ascorbic acid was detected at 254 nm.

3.4.3.3 Carotenoids extraction and assay

The broccoli seeds sample (10 g) was extracted successively (usually three times) with 40 mL ethanol-hexane (1:1), followed by filtration in a glass sintered funnel. The combined filtrates were transferred to a separatory funnel containing 25 mL hexane and 20 mL water. After gentle shaking for 30 to 60s, the liquid phases were allowed to separate. The aqueous phase was drawn into a second separatory funnel and re-extracted three times with 40 mL portions of ethanol-hexane (1:1). The combined hexane extracts were washed three times with purified water, collected, dried over anhydrous Na_2SO_4 and concentrated in a rotary evaporator

(40° C), the volume being adjusted to 25 mL. Samples were separated on a Novapak 5 μm RP-18 column (150 x 3.9 mm) by an HPLC HP 1090 equipped with a photodiode array detector. The mobile phase consisted of acetonitrile-chloroform (9:1). The flow rate of mobile phase was 1.0 mL/min. The content of carotenoids was expressed as milligramm β-carotene equivalents per 100g DW at 450 nm.

3.4.3.4 Tocopherols extraction and assay

The broccoli seeds were ground (10 g), saponified and protected from light at room temperature over 2 h with constant stirring in an alcoholic potassium hydroxide solution (KOH) plus ascorbic acid to avoid oxidation of the liposoluble vitamins. The composition of the reagent was as follows: 50 mL of ethanol, 10 mL of 0.1% aqueous ascorbic acid, 10 mL of aqueous 40% KOH and 25 mL of water. The analytical samples were then extracted with n-hexane (2x25 mL) and the extracts were washed with water (2x10 mL). The organic phase was removed by evaporation in a rotary evaporator under vacuum at 50° C. The residue was redissolved in methanol (50 mL) and filtered through Sartolon polyamide membranes filter (Sartorius, Germany) with a specific pore size of 0.45 μm to clean the extracts before their injections into the chromatographic system. A 290 nm wavelength kit was fitted into the detector. A 250 x 4.6 mm i.d. LiChrosorb RP-18 (5 μm) column was used with 2.5 mM acetic acid-sodium acetate in methanol-water (97:3) as mobile phase. The flow rate set at was 1.0 mL/min.

3.4.3.5 Method validation

The validation characteristics of HPLC method for individual antioxidant activity were evaluated according to the method described in section 3.3.4 (method validation), page 49.

3.5 Statistical method analysis

Analysis of variance (ANOVA) was performed on data to identify the significant differences in glucosinoaltes and antioxidant capacity among genotypes by a statistical program SPSS version 10.0.7 for windows, SPSS Inc., USA. The least significant differences (Fisher's LSD) were determined among broccoi genotypes at $p = 0.05$. Pearson's correlation coefficients were calculated using means of each assayed in triplicate samples from each broccoli genotype analytical data.