Quantitative Determination of the Glucosinolates Sinigrin and Progoitrin by Specific Antibody ELISA Assays in Brussels Sprouts

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Glucosinolates from Brussels sprout samples were extracted using an effective concentration of 2% phosphoric acid followed by a neutralization step and heat treatment for removal of inactivated protein. The (potentially) bitter glucosinolates sinigrin and progoitrin were found to be stable during this new extraction protocol. Antisera, as raised against hemisuccinate-linked glucosinolate conjugates, were very specific in sandwich ELISA assays for their corresponding substrates. The ELISA assays showed maximally 7.4% cross-reactivity to other aliphatic glucosinolates and were log−linear from the nM to μM range. In comparison to the standard HPLC method, the sinigrin and progoitrin ELISA respectively slightly and considerably overestimate the actual content of these glucosinolates. The progoitrin content of samples as determined either with the ELISA assay or by HPLC, however, is highly correlated ($r^2 = 0.92$, $n = 12$, $p < 0.01$), suggesting that the former assay is also applicable for the screening of the progoitrin content in Brussels sprout samples.

Keywords: *Sinigrin; progoitrin; glucosinolates; ELISA; phosphoric acid extraction; Brussels sprouts*

INTRODUCTION

The taste and consumption quality of edible parts and seeds from cruciferous plants, such as Brussels sprouts, are significantly determined by glucosinolates, a group of thioglucosides with health-promoting or toxic properties for animals and humans. More than 90 glucosinolates have already been identified, which can be categorized in three groups consisting of aliphatic, aromatic and indole glucosinolates (Fenwick et al., 1983a, 1983b) depending on the chemical structure of their functional side chain. In crucifers, glucosinolates coexist with the enzyme thioglucosidase (E.C. 3.2.3.1), an enzyme with specific catabolic activity toward glucosinolates, which is located in isolated cells of (un)differentiated tissues (Thangstad et al., 1990) strictly separated from its substrates. After disrupture of the tissue, the glucosinolates come into contact with thioglucosidase (also known by the trivial name myrosinase) and are converted into glucose, sulfate, and their corresponding isothiocyanates, thiocyanates, and nitriles (Uda and Maeda, 1986; Springett and Adams, 1988). Thioglucosidase activity differs among the various crucifers (Springett and Adams, 1989; Palmieri et al., 1987; Wilkinson et al., 1984; Yen and Wei, 1993). Considerable variation in activity is also observed among cultivars of the same species and plant parts of specific cultivars (Pocock et al., 1987).

Extraction of glucosinolates from crucifer tissues is a difficult process, since glucosinolates are always accompanied by thioglucosidase and homogenization without inactivation of the enzyme always leads to a rapid loss in yield. For this reason many extraction protocols have been developed in which glucosinolates are extracted in various (boiling) organic solvents (Fenwick, 1984; Finnigan and Lewis, 1988; Hanley et al., 1983; McGregor et al., 1983; Peterka and Fenwick, 1988; Shasidi et al., 1990; Visentin et al., 1992) with the aim of inactivating thioglucosidase during the extraction process and dissolving all glucosinolates with high recovery.

The diversity in glucosinolate content and composition in crucifers on the one hand and the isozyme patterns of thioglucosidase on the other hand suggest that glucosinolates play a dominant role in the flavor characteristics of cruciferous species. Glucosinolates and their corresponding breakdown products are indeed characterized as the principles of aroma and flavor in cruciferous vegetables (Fenwick et al., 1983a). The content and distribution of glucosinolates have been studied in the edible parts of almost all species of commercial Brassica plants (Carlson et al., 1981; Daxenbichler et al., 1979; Heaney and Fenwick, 1980a, 1980b; Hill et al., 1987; Lewis and Fenwick, 1987, 1988; Sones et al., 1984; VanEtten et al., 1976, 1980) with the utilization of many different analytical techniques, which are reviewed by McGregor et al. (1983). More sophisticated techniques have since been developed for the determination of total glucosinolate content in samples: X-ray fluorescence (Schnug and Haneklaus, 1988), reflectance (Tholen et al., 1993), autoanalyzer (Smith et al., 1985), soluble and immobilized enzymes (Kuan et al., 1986), or NIR spectroscopy (Biston et al., 1988). The determination of single glucosinolates has also been optimized; e.g., glucosinolates now can be analyzed by high-performance liquid chromatography (HPLC) without desulfatation (Bjorkqvist and Hase, 1988). Indole glucosinolates can be determined by spectrophotometry after a simple derivatization step (Thies, 1990).

The determinations were, however, always restricted to a small number of cultivars, owing to time-consuming sample preparations and analysis of the glucosinolates

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by gas liquid chromatography (GLC) or high-performance liquid chromatography.

Enzyme-linked immunosorbent assays (ELISAs) are very suitable for the analysis of the glucosinolate content in large numbers of samples. ELISAs are particularly useful for the initial screening of the glucosinolate content and composition in hundreds of parental lines, cultivars, and plants of segregating populations as present in breeding programs. For such a purpose conventional analytical techniques such as GC and HPLC are too time-consuming to allow an efficient selection during the selection season in plant breeding. An ELISA assay was published for the determination of alkenyl glucosinolates in series of seed samples by Hassan et al. (1988). The polyclonal antisera raised against a sinigrin-BSA conjugate showed a high cross-reactivity for progoitrin and gluconapin and were, therefore, not specific for the quantitative determination of sinigrin.

Particular Brussels sprout cultivars develop a pronounced bitter taste, which is evident in both fresh as well as frozen sprouts (Bedford, 1989), probably because of the presence of certain glucosinolates. The glucosinolate content of Brussels sprout cultivars consists mainly of sinigrin (allylglucosinolate), progoitrin (2-hydroxy-3-butenylglucosinolate), gluconapin (3-butenylglucosinolate) and minor amounts of indolglucosinolates (Carlson et al., 1987; Heaney and Fenwick, 1980a, 1980b; Heaney et al., 1983).

Fenwick et al. (1983b) and Griffiths and Fenwick (1984) found sinigrin and progoitrin to be the compounds that cause bitterness in buttons of Brussels sprouts. Sinigrin had a bitter taste as an intact glucosinolate, while progoitrin was intensely bitter after enzymatic decomposition to goitrin ((−)5-vinyloxazolidine-2-thione).

In order to study the influence of the (potentially) bitter glucosinolates on the taste preference of consumers and to select for a lower content in our breeding program, large-scale convenient extraction methods and assays for glucosinolates were necessary.

In this paper, a simple sample preparation method for glucosinolates and specific ELISA assays for sinigrin and progoitrin are presented for the screening of breeding programs of Brussels sprouts for both glucosinolates.

MATERIALS AND METHODS

(A) ELISA Assays for Sinigrin and Progoitrin. *Chemicals.* Radioimmunoassay-grade bovine serum albumin (BSA), ovalbumin (electrophoretic 99% pure), goat:antirabbit IgG−alkaline phosphatase conjugate (IgG−APase), Sigma 104 phosphatase substrate (PNP), *N*-hydroxysuccinimide, *N*,*N*′-dicyclohexylcarbodiimide, glucocheirolin, and sinigrin were obtained from Sigma Chemical Co. (St. Louis, MO). Gluconapin and progoitrin were a kind gift of Dr. Brian Hanley and Dr. Roger Fenwick of the AFRC in Norwich, U.K. Nunc class I plates were purchased from Nunc Inter-med., Copenhagen, Denmark. Standard reagents (pro analyze quality), which have been used in buffers, and organic solvents (purity, >99%) all were obtained from Sigma, Chemical Co. (St. Louis, MO).

Buffers. The coating buffer used was 100 mM sodium bicarbonate (pH = 9.6).

For phosphate-buffered saline (PBS), 1 L of 150 mM PBS contained 8 g NaCl, 1.15 g Na_2HPO_4:$2H_2O$, 200 mg KCl, and 200 mg KH_2PO_4, adjusted to pH = 7.2 with 2 N NaOH. PBS, pH = 7.6 was used as reaction buffer for the synthesis of sinigrin and progoitrin conjugates.

The washing buffer (PBST) was 0.05% (v/v) (500 μL/L) Tween 20 in PBS.

The antibody diluent (PBSTO) was PBST buffer containing 0.01% (w/v) ovalbumin.

The alkaline phosphate substrate consisted of 100 mM glycine, 1 mM $ZnCl_2$, 1.5 mM $MgCl_2$:$6H_2O$, and 1 mg/mL Sigma 104, pH = 9.7.

Synthesis of Sinigrin−BSA, Sinigrin−Ovalbumin, Progoitrin−BSA, and Progoitrin−Ovalbumin Conjugate. Sinigrin−BSA conjugate is prepared according to Hassan et al. (1988) with some modifications. Samples of 130 mg sinigrin and 152 mg succinic anhydride were dissolved in 1 mL dimethylformamide and 26 μL pyridin and allowed to react at room temperature for 5 days in a closed glass reaction tube.

The sinigrin hemisuccinate was crystallized by addition of 10 mL ether and collected by centrifugation (table centrifuge, 2000*g*). The precipitate was dissolved in 1 mL methanol and subsequently precipitated again by the addition of 10 mL diethylether and centrifuged (table centrifuge, 4000*g*). Thereafter, the precipitate was dissolved in 1 mL methanol and freeze-dried overnight.

The freeze-dried sinigrin hemisuccinate, 83 mg of *N*,*N*′-dicyclohexylcarbodiimide (DCCD), and 90 mg of *N*-hydroxysuccinimide were dissolved in 1.5 mL of dimethylformamide in order to synthesize the activated *N*-hydroxysuccinimide ester of sinigrin. The solution was allowed to react for 12 h at 4 °C without any mixing. During this period, crystals of dicyclohexylurea were formed, which were removed by loading the solution on a disposable column equipped with a 4-μm porous glass filter followed by centrifugation (table centrifuge, 4000*g*). The clear filtrate (1.25 mL) was divided into two equal portions of 625 μL for the preparation of sinigrin−BSA and sinigrin−ovalbumin conjugates.

A quantity of 625 μL of solution of activated *N*-hydroxysuccinimide ester of sinigrin was added to either a solution of 21.2 mg BSA or 42 mg ovalbumine in 2 mL PBS buffer pH = 7.6 and mixed gently for 24 h at 4 °C using a mechanical stirrer. In both mixtures a white sediment was formed immediately during the first hours. The crude sinigrin−BSA and sinigrin−ovalbumin conjugates were dialyzed three times for 12 h in dialysis tubing against 1 L of demineralized water (Barnstead Nanopure apparatus, Boston, MA) to remove unreacted chemicals. The dialyzed conjugates were freeze-dried and stored refrigerated (4 °C) in closed Eppendorf reaction vessels. The yield was 42 mg of sinigrin−BSA and 60 mg of sinigrin−ovalbumin conjugate.

The epitope density was determined using the difference in weight between the synthesized conjugate and the initial weight of BSA or ovalbumin. The following assumptions were made: molecular weight of BSA = 64 kD, free amino groups of BSA = 61, molecular weight of ovalbumin = 45 kD, free amino groups of ovalbumin = 20, molecular weight of sinigrin moiety = 522.

The preparation of progoitrin conjugates was based on the same principle as for the sinigrin conjugates, but on a smaller scale. Samples of 50 mg progoitrin, 59 mg of succinic anhydride, and 20 μL of pyridine were incubated in 770 μL of dimethylformamide for 5 days at room temperature. The isolation of the progoitrin hemisuccinate was identical to the isolation procedure of the sinigrin hemisuccinate. In contrast to the sinigrin hemisuccinate that had a white color, the progoitrin hemisuccinate had a typical brownish color. Samples of 52 mg of progoitrin hemisuccinate, 30 mg of *N*-hydroxysuccinimide, and 27.6 mg of *N*,*N*′-dicyclohexylcarbodiimide were dissolved in 500 μL of dimethylformamide and incubated overnight at 4 °C without any mixing. During the incubation, crystals of dicyclohexylurea were formed. The dicyclohexylurea was removed by centrifugation. The supernatant (750 μL) contained the active N-hydroxysuccinimide ester of progoitrin. A quantity of 185 μL was added to 21 mg ovalbumine dissolved in 1 mL PBS buffer, pH = 7.6. A quantity of 555 μL was added to 30 mg BSA dissolved in 3 mL PBS buffer, pH = 7.6. Both solutions were mixed gently with a mechanic stirrer for 24 h at 4 °C. The crude progoitrin conjugates were dialyzed three times for 12 h in dialysis tubing against 1 L of demineralized water to remove unreacted chemicals, freeze-

dried and stored refrigerated (4 °C) in closed Eppendorf vessels. Both conjugates had a typical brownish color.

NMR Spectroscopy of the Progoitrin Hemisuccinate. The glucosinolate moiety is coupled to BSA or ovalbumin by means of an active *N*-hydroxysuccinimide ester attached on the glucose molecule of the glucosinolate. Progoitrin not only contains hydroxyl groups on the glucose moiety but also contains a hydroxyl group on C_3 of the aliphatic side chain. Esterification of this specific hydroxyl group will destroy the specific antigenic quality of the progoitrin molecule. NMR studies were done by Dr. C. Kruk, Department of Organic Chemistry at the University of Amsterdam to check whether the hydroxyl group of the progoitrin hemisuccinate was esterified. Two-dimensional $^{13}C-^{1}H$ correlation NMR, attached proton test (APT) ^{13}C NMR, and proton NMR confirmed that the hydroxyl group on C_3 of the aliphatic side chain of progoitrin was not esterified.

Immunization of Rabbits. Two rabbits were immunized by Dakopatts (Glostrup, Denmark) with either the sinigrin−BSA or the progoitrin−BSA conjugate. The rabbits were immunized with 1 mL of 0.1 M NaCl/Freund's complete adjuvant 1:1 (v/v) containing 1.32 mg of sinigrin-BSA or the progoitrin−BSA conjugate. Booster injections were applied every 2 weeks following the first immunization. The first bleeding was 68 days after the initial immunization. From every bleeding, a volume of 20−25 mL serum was obtained.

Thereafter, the rabbits were given a booster injection every 4 weeks and bled 12 days later. Thus, three bleedings of 20−25 mL antiserum were obtained from both rabbits. The sera were isolated from the blood by centrifugation and stored after the addition of 0.1% sodiumazide at −70 °C.

Coating, Titers, Specificity, Cross-Reactivity, Enzyme Assay. The coating of immunoplates, the determination of titers, specificity, and cross-reactivity of the obtained antisera, and the enzyme assay (alkaline phosphatase) were done according to standard procedures as described by Hassan et al. (1988). Cross-reactivity is defined as (sinigrin or progoitrin concentration for 50% inhibition)/(glucosinolate concentration for 50% inhibition) × 100.

(B) Extraction of Glucosinolates with Phosphoric Acid. *Brussels Sprouts and Sample Preparation.* Hybrids of Brussels sprouts were grown under standard conditions in the trial field for cultivar assessment (for the evaluation of postharvest traits such as uniformity, storage quality, flavor, etc.) as performed by Novartis Seeds BV in De Schermer, The Netherlands.

After they were harvested duplicate samples of 200 g were homogenized with 200 mL of 4 v/v % phosphoric acid in a Braun knife homogenator (Braun, Germany) for 1 min. Due to the 1:1 ratio of sprouts and 4% phosphoric acid (on a weight basis) is the effective phosphoric acid concentration during the homogenization step, 2%. After the homogenization step, the obtained slurry was transferred to a funnel that was positioned on a beaker of glass and equipped with a paper filter (Schleicher & Schuell, Germany) to separate the glucosinolate-containing fluid from solid cell-wall material. Of each obtained filtrate a duplicate sample of 1 mL was transferred to a 96-well plate (Micronic). Filled 96-well plates, containing 48 1-mL samples in duplicate, subsequently were centrifuged in a table centrifuge (Jouan, France, 4000*g*).

After centrifugation, 500 μL of clear supernatant was transferred to a new 96-well plate and neutralized with approximately 55 μL of 5 N KOH at pH = 7.0.

After neutralization the plates were centrifuged (Jouan, France, 4000*g*) to remove the calcium phosphate precipitate, since sprouts were found to contain a high inorganic calcium content. The supernatant was transferred to a new microtiter plate, sealed, covered with a lid, and heated in a boiling water bath for 10 min to denature dissolved proteins and destroy putative residual enzyme activity that might recover after neutralization at pH = 7.0. Thereafter, the plates were cooled to room temperature by floating on water and again centrifuged to remove the precipitated sediments. Clear samples were stored in sealed microtiterplates at −20 °C until determination of the content of glucosinolates. By use of this sample preparation protocol, 100 samples could be prepared in duplicate per day.

Stability of Sinigrin and Progoitrin in 2% Phosphoric Acid and during Boiling. A good extraction solvent extracts glucosinolates efficiently and guarantees the stability of the glucosinolates during the extraction process. Phosphoric acid efficiently extracts amino acids, sugars, and other water-soluble metabolites from plant tissues. The stability of sinigrin and progoitrin in 2% phosphoric acid, the actual concentration during the extraction of glucosinolates, was studied in a time course of up to 4 h, a convenient period for the preparation of 100 sprout samples in duplicate. Sinigrin and progoitrin were dissolved at a concentration of 2 mM in 5 mL of 2% phosphoric acid or in 5 mL of 350 mM potassium phosphate buffer (equimolar concentration phosphate), pH = 7.0, as a control. Every hour a 500-μL sample was taken from the glucosinolate solutions and quickly neutralized at pH = 7.0 with 5 N KOH. A 2 mM concentration of glucose in 2% phosphoric acid and 350 mM potassium phosphate was included in the experiment as a positive and negative control.

The stability of the glucosinolates was determined in terms of split-off glucose due to acidic hydrolysis using an enzymatic assay for glucose according to Boehringer Mannheim (1989) and expressed as percentage recovery of the initial concentration of 2 mM.

Phosphoric acid at a concentration of 2% effectively inactivates myrosinase and other enzymes during the extraction process but does not precipitate proteins as effective as perchloric acid or trichloroacetic acid (van Doorn et al., 1989). To precipitate inactivated but still dissolved proteins a cooking treatment of 10 min at 100 °C was found to be efficient. The stability of sinigrin and progoitrin was determined in samples of 2 mM of both glucosinolates in 2% phosphoric acid after neutralization and expressed as presented before.

(C) Determination of Sinigrin and Progoitrin in Sprout Samples. *Precision of the ELISA Assays.* Both ELISA assays for sinigrin and progoitrin were tested for precision using an HPLC method for intact glucosinolates as a reference method (Bjorkqvist and Hase, 1988). Brussels sprouts samples were determined for the content of sinigrin and progoitrin both with ELISA assays and by HPLC.

HPLC Reference Method for the Determination of Glucosinolates. Samples of Brussels sprouts, as prepared with the phosphoric acid extraction method, were determined for the content of intact sinigrin and progoitrin by isocratic reversed-phase ion-pair chromatography. The HPLC system (Waters) was equipped with a C_{18} reversed-phase column and was run with an eluent that was composed of 100 mM ammonium acetate, 10 mM tetrabutylammonium chloride, and 5% acetonitrile (pH = 7.0) at a flow of 2 mL/min. The glucosinolates were detected at 235 nm. The influence of the sample matrix on the % recovery of sinigrin and progoitrin was studied with internal standards of both glucosinolates at a specific dilution of the samples. The recovery of both glucosinolates also was studied in a series of dilutions for specific samples.

Determination of the Total Sum of Glucosinolates. The total sum of glucosinolates was determined according to the modified glucose release method of Van Doorn et al. (1997).

RESULTS AND DISCUSSION

Breeding for secondary metabolites that have an impact on taste and quality of vegetables is often restricted by time-consuming sample preparation and determination protocols.

In this report, the development of a relatively quick sample preparation method and two highly specific polyclonal antisera is reported that allows the large-scale and quick determination of the glucosinolates sinigrin and progoitrin in the Brussels sprouts breeding program.

ELISA Assays for Sinigrin and Progoitrin. Specific antibodies are a nice analytical tool for the quan-

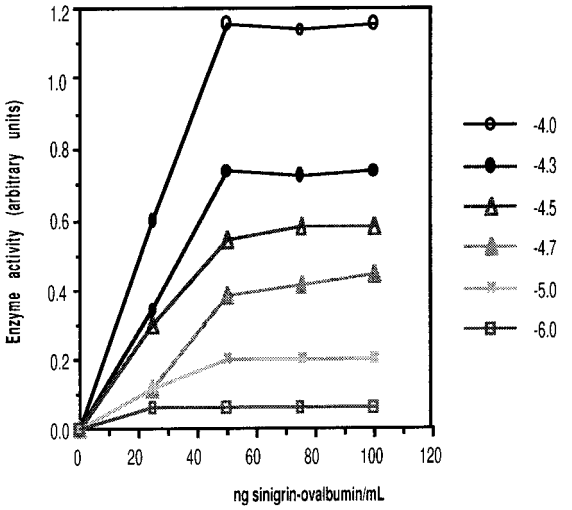

Figure 1. Checkerboard titration of four concentrations of sinigrin−ovalbumin antigen against six different dilutions of antiserum raised against sinigrin. Antisera dilutions are log-transformed. Incubation time is 2 h at room temperature. Observed enzyme activity originates from alkaline phosphatase labeled mouse anti rabbit antiserum (see Materials and Methods)

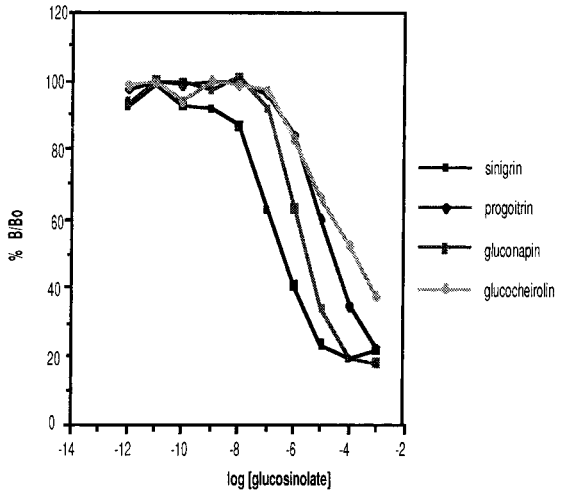

Figure 2. Competitive ELISA using anti-sinigrin serum. Plates were coated with 50 ng of sinigrin−ovalbumin/mL and antiserum was 10^4-times-diluted. The glucosinolate concentration was varied between 1 pM and 1 mM for the glucosinolates sinigrin, progoitrin, gluconapin, and glucocheirolin. The incubation time was 2 h at room temperature.

titative determination of secondary metabolites such as glucosinolates. Antibodies are applicable for the screening of metabolites in samples by immunoassays. Antisera for immuno assays need to have sufficient specificity and a high titer. The prepared sinigrin−BSA and sinigrin−ovalbumin were very poorly soluble in the coating buffer. The poor solubility of the conjugates seems to be due to the high degree of conjugation since all free amino groups of the sinigrin−BSA and sinigrin−ovalbumin conjugates were occupied by the active ester of sinigrin. No protein was detected in the conjugates with an amino-group-directed protein assay (data not shown). Immunization of two rabbits gave two different antisera with regard to the titer and specificity.

One of the two rabbits produced a highly specific antiserum against sinigrin as specified in this report. The antibody titer was determined with a dilution between 10^3- and 10^6-fold on plates coated with 50 ng of sinigrin−ovalbumin/mL. The log of dilution of antiserum and the extinction at 410 nm were linearly related at dilutions between 10^3- and 10^5-fold. The antibody titer did not change during the three successive bleedings (not shown). Nonspecific background extinction was below 0.1 for all dilutions, indicating the high selectivity of the antibodies for the sinigrin−ovalbumin conjugate.

Figure 1 shows the checkerboard titration of four different concentrations of sinigrin−ovalbumin antigen against six different dilutions of antiserum. A concentration of 50 ng of sinigrin−ovalbumin/mL was the minimal quantity of antigen leading to an optimal extinction at all antiserum dilutions. A 10^4-times-diluted antiserum gave one extinction unit within 2 h and was chosen to test the selectivity of the serum for sinigrin and the cross-reactivity for other glucosinolates.

The typical standard curve for competitive inhibition of the sinigrin ELISA was log−linear between 10 nM and 10 μM sinigrin, an appreciable wide range to determine sinigrin concentrations in samples.

The cross-reactivity of the antiserum against the glucosinolates gluconapin, progoitrin, and glucocheirolin is shown in Figure 2. The antiserum turned out to be very specific for sinigrin; only gluconapin had a cross-reactivity of about 7.4%. The cross-reactivity of the glucosinolates progoitrin and glucocheirolin was negligible. The specificity of the antiserum for sinigrin increased from the first to the third bleeding, since the cross-reactivity for gluconapin decreased from 13.0 to 10.0 to 7.4% in the three successive bleedings, respectively.

The progoitrin−BSA and progoitrin−ovalbumin conjugate had the same properties as the sinigrin conjugates. All free amino groups in both conjugates were almost completely occupied by progoitrin moieties. After immunization of two rabbits, one rabbit raised a highly specific antiserum against progoitrin. The titer of this serum was comparable with the titers obtained for the antisera against sinigrin. The second bleeding had almost the same titer. The rabbit died after the second bleeding. A checkerboard titration showed that plates were coated optimally with 50 ng of progoitrin−ovalbumin/mL (not shown). Plates coated with 50 ng of progoitrin−ovalbumin/mL and incubated with 10^4-times-diluted antiserum produced one extinction (410 nm) unit within 1 h. The background extinction was 0.3 (30% on the B/Bo scale), thus somewhat higher than for the sinigrin ELISA. The typical standard curve for the competitive inhibition of the progoitrin ELISA for the corresponding substrate progoitrin was log−linear between 10 nM and 100 μM progoitrin.

The cross-reactivity of the antiserum against the glucosinolates sinigrin, gluconapin, and glucocheirolin is shown in Figure 3. The antiserum of the second bleeding had a cross-reactivity of 4.6% for sinigrin, gluconapin, and glucocheirolin.

Hassan et al. (1988) reported antisera against sinigrin which had a high degree of cross-reactivity to other aliphatic glucosinolates. The first serum showed a cross-reactivity of 100% against the glucosinolates gluconapin and progoitrin. The second serum showed a cross-reactivity of 100 and 56% against, respectively, gluconapin and progoitrin. In contrast, our approach yielded highly specific antisera against sinigrin and progoitrin. In both cases, one of two rabbits immunized with sinigrin−BSA or progoitrin−BSA conjugate raised a highly specific polyclonal antiserum.

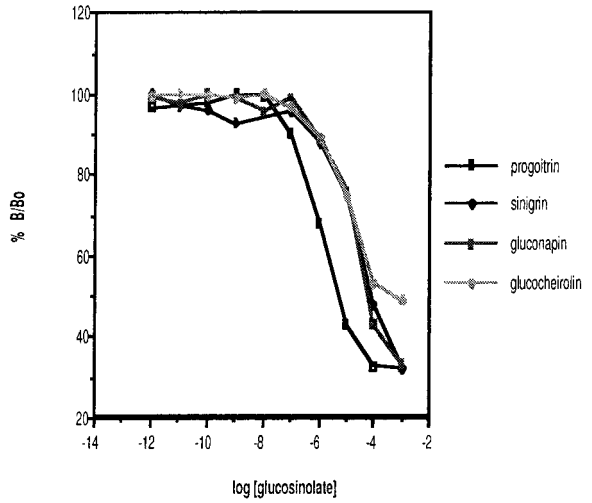

Figure 3. Competitive ELISA using the anti-progoitrin serum. Plates were coated with 50 ng of progoitrin−ovalbumin/mL and anti-progoitrin serum was 10^4-times-diluted. The glucosinolate concentration was varied between 1 pM and 1 mM for the glucosinolates progoitrin, sinigrin, gluconapin, and glucocheirolin. The incubation time was 1 h at room temperature.

The sinigrin−BSA and sinigrin−ovalbumin conjugates of Hassan et al. (1988) were partially conjugated. Sixty-one and 65% of the free amino groups of BSA and ovalbumin, respectively, were occupied by sinigrin. Our conjugates were almost completely conjugated, since no free amino groups were detectable with amino-group-directed protein assays. This difference in conjugation rate might explain the higher specificity of our antiserum to sinigrin and progoitrin. Coupled to this high specificity, the antisera show a very low cross-reactivity against other glucosinolates that might equally be the result of very uniform "high-density" conjugates of sinigrin and progoitrin−BSA.

The antiserum against sinigrin had a cross-reactivity of 7.4% for gluconapin, probably because gluconapin is very related to sinigrin, differing only in a $-CH_2$ group in the side chain of the glucosinolate.

Quality and Reliability of the Acidic-Sample Preparation Method. It is essential that the extraction process of glucosinolates is quick and efficient because of their rapid degradation in disrupted tissues (see Introduction). We developed a method in which 4 v/v % phosphoric acid is used as an extractant for the preparation of glucosinolate samples from Brussels sprout buttons. Sprouts and extractant were used in a 1:1 ratio during sample preparation, resulting in an effective concentration of 2% phosphoric acid in the homogenate. The sample preparation method with phosphoric acid was tested for effectiveness at three critical essential steps: (1) the acidic extraction step for the inactivation of thioglucosidase and the extraction of glucosinolates at room temperature, (2) the neutralization step to an optimal pH = 7.0 for glucosinolates, and (3) the boiling step to inactivate and remove residual proteins.

The stability of 2 mM sinigrin and progoitrin was studied in 2% phosphoric acid, the actual concentration of phosphoric acid in the homogenate during the extraction step, for a period up to 4 h, a convenient time to prepare sprout samples on a large scale. Both glucosinolates were found to be stable in this acidic environment during the whole period. A recovery of 99 ± 1% (n = 2) was observed after determination of the glucosinolates by means of the glucose-release method of Van Doorn et al. (1997). Boiling of neutralized 2 mM sinigrin and progoitrin solutions for a period of 10 min gave the same recovery for both glucosinolates (data not shown).

Within Brussels sprout samples, a sinigrin and progoitrin content of maximally 6 and 4 g/kg fresh weight, respectively, have been found (van Doorn et al., 1997), equivalent to concentrations of up to 10 mM of the individual glucosinolates in neutralized sprouts samples according to our extraction protocol.

An internal standard of 2 mM sinigrin and progoitrin was added to samples of sprout buttons during the homogenization step with phosphoric acid, and afterwards the recovery of both glucosinolates was determined by an assay for the total glucosinolate content by the glucose release assay. The results indicated that sinigrin and progoitrin were stable in the acidic matrix during the extraction process (data not shown).

From the results it can be concluded that sinigrin and progoitrin can be effectively extracted from fresh sprout samples without any degradation of these glucosinolates during the successive sample preparation steps, e.g., the homogenization in 2% phosphoric acid, the neutralization with KOH, and the boiling to remove proteins. This method, therefore, was applied for the large-scale preparation of sprout samples.

The method yields samples that contain a sinigrin and progoitrin content that exceeds the values reported previously (Fenwick et al., 1983b; Heaney et al., 1983), which might be indicative that the phosphoric acid extraction procedure has a better recovery of glucosinolates. However, new, improved cultivars have been introduced on the market since then, which is an alternative explanation for the increased content of sinigrin and progoitrin of cultivars in this study. The extraction protocol has clear advantages in comparison to the currently used methods in which glucosinolates are extracted in boiling organic solvents. The acidic inactivation of myrosinase allows the large-scale preparation of samples with relatively simple homogenators. Time-consuming steps such as vacuum evaporation, the addition of internal standards, and quantitative collection of glucosinolates are not necessary in our protocol.

Determination of Intact Sinigrin and Progoitrin by HPLC. The determination of glucosinolates by HPLC is routinely conducted on glucosinolate samples that have been obtained by classical isolation methods involving boiling with organic solvents. Such samples contain glucosinolates in a relatively clean matrix in which also other organic-solvent-soluble compounds are dissolved. In the case of analysis of desulfoglucosinolates, the matrix of the samples is even more free of disturbing plant metabolites, since the matrix is then only composed of sulfatase in an acetic acid buffer. The influence of the complex sample matrix, as obtained after the phosphoric acid extraction procedure, on the determination of glucosinolates by isocratic ion-pair reversed-phase HPLC was tested (see Materials and Methods). Progoitrin and sinigrin elute from the column after 1.5 and 2.0 min, respectively, under the specified conditions.

The peak area for sinigrin and progoitrin are found to be highly linearly related to the injected sample volume for 100-times-diluted samples. Linear relationships (n = 3, $r^2 > 0.99$) were observed between the

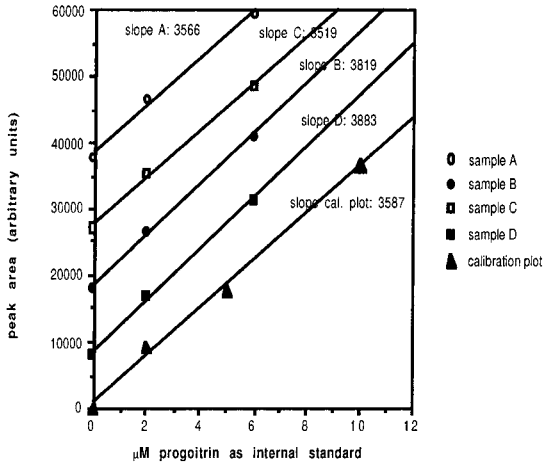

Figure 4. Recovery of internal standards of progoitrin in 100-fold-diluted sprout samples. Internal standards were added as a concentration of 0, 2, and 6 μM glucosinolate. Peak area is given in arbitrary units of the detector at 0.002 AUFS.

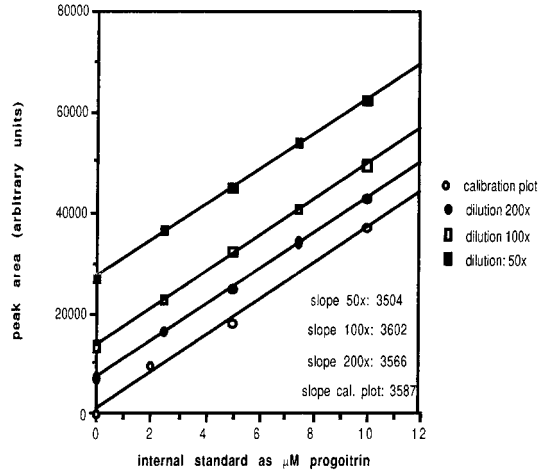

Figure 5. Recovery of internal standards of progoitrin in sprout samples at variable dilutions. Internal standards were added as a concentration of 0, 2.5, 5.0, 7.5 and 10 μM glucosinolate. Peak area is given in arbitrary units of the detector at 0.002 AUFS.

sample volume and the peak area for up to 200 μL of injected sample with an intercept for the lines close to zero. Comparable figures were obtained at other dilutions. It was concluded that the sample matrix had no influence on the quantification of both glucosinolates using ion-pair HPLC.

In Figure 4, four 100-times-diluted sprout samples are spiked with an internal standard of 0, 2, and 6 μM of progoitrin. The figure clearly shows that the different samples and the concentration of progoitrin in the samples had no influence on the recovery of the internal standards. The slopes that were calculated from the relationships between the concentrations of the internal standard and the glucosinolate in the spiked samples were almost equal to the slope of the calibration plot of the internal standard in water. The average slope of samples was only 2.8% higher than the slope of the calibration plot.

Comparable recovery figures were obtained when sinigrin was spiked in these four samples (data not shown).

In Figure 5, a sample is diluted 50, 100, and 200 times and spiked with 0, 2.5, 5, 7.5, and 10 μM progoitrin, respectively, to study whether the dilution rate has an influence on the recovery of added internal standard. The recovery of the spiked internal standards, expressed as the slope from the relationship between the concentration of the internal standard and the glucosinolate content in the spiked samples, is on average almost 100% for progoitrin (−0.8%). Again, comparable recovery figures were obtained when sinigrin was spiked in samples with a variable dilution rate (data not shown).

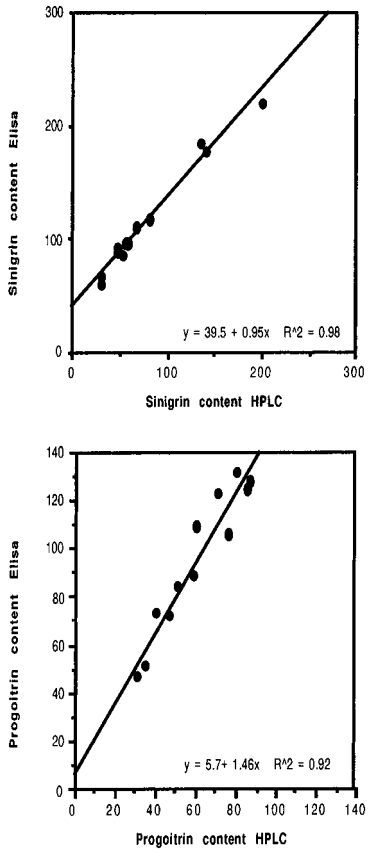

Figure 6. Correlative studies to compare HPLC and ELISA assays for the glucosinolates sinigrin (upper graph) and progoitrin (lower graph). The determination of the content of both glucosinolates was conducted as described under Materials and Methods. The content of both glucosinolates is expressed in mg/100 g.

From the results it is clear that samples, which have been prepared with the phosphoric acid extraction protocol, can easily be determined with ion-pair RP HPLC. The content of progoitrin and sinigrin is linearly related to the dilution rate; internal standards show 100% recovery at variable sample dilutions and at variable concentrations of both glucosinolates.

Sinigrin and Progoitrin Content of Hybrids of Brussels Sprouts: Comparison between the ELISA Assays and the HPLC Method. The antisera were applied on samples of sprouts from various cultivars that have been prepared by phosphoric acid extraction method. The samples were also determined for the content of both glucosinolates by the HPLC method to compare the methods with each other. The sinigrin and progoitrin content of samples could be determined with the ELISA assays using a dilution factor of 1000 to obtain concentrations of sinigrin and progoitrin in the linear concentration range of the calibration plot.

In Figure 6 the sinigrin content in samples of 12 different cultivars as determined by HPLC and ELISA was correlated (top graph). It is shown that the sample preparation method yields samples that contain a

sinigrin content from 40 to 250 mg/100 g fresh weight. From the equation that describes the relationship between the content determined by HPLC and antibody assay, it is clear that both methods are almost comparable for aspects such as sensitivity (slope = 0.95) and reliability ($r^2 = 0.92$). Both methods will yield about the same sinigrin content in samples.

In the lower graph of Figure 6 the progoitrin content of the same samples as determined by ELISA and HPLC is correlated. The highest progoitrin content in the samples was, depending on the method, about 90 or 130 mg/100 g fresh weight.

The equation that describes the relationship between both methods shows that the ELISA assay for progoitrin in all cases gives a higher progoitrin content in samples than the HPLC method. The progoitrin content as obtained with either the ELISA or HPLC method is relatively very reliable ($r^2 = 0.92$, $n = 12$, $p < 0.01$).

The higher progoitrin content in the ELISA assay might be due to a matrix effect of the samples. Progoitrin is a relatively polar glucosinolate that is hydroxylated on the alkyl group. A polar matrix, which has, for instance, a high phosphate concentration, might decrease the binding of antibodies to the coated progoitrin−ovalbumin conjugate during the competitive binding step of the ELISA assay. Thus, an overestimation of the progoitrin content occurs that is based on an artifact. The overestimation of the progoitrin content in the ELISA assay also might be explained by the quality of the polyclonal antiserum. The possibility cannot be excluded that (part of) the polyclonal progoitrin-specific antibodies bind to an epitope that is composed of two conjugated progoitrin molecules.

The observed difference between the ELISA and HPLC method is about 50% but proportionally comparable for samples that differ in the content of progoitrin, allowing the correction for this phenomenon by extrapolation.

Time-consuming glucosinolate determinations by HPLC can be replaced by rapid, reliable, and quantitative ELISA assays. With ELISA assays, the number of samples no longer forms a limitation to the determination of glucosinolates. Both antisera proved to be perfect tools for the determination of sinigrin and progoitrin content in edible parts of cruciferous vegetables and can also be applied for the determination of these glucosinolates in other types of samples such as seeds. The application of specific antisera for the analysis of glucosinolates is of significant importance to the study of glucosinolate metabolism and inheritance in breeding programs of Brassica vegetables.

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LITERATURE CITED

Bedford, L. V. Sensory appraisal of Brussels sprouts for the freezing industry. *Acta Hortic.* **1989**, *244*, 57−64.

Biston, R.; Dardenne, P.; Cwikowski, M.; Marlier, M.; Severin, M.; Wathelet, J.-P. Fast analysis of rapeseed glucosinolates by near infrared reflectance spectroscopy. *JAOCS, J. Am. Oil Chem. Soc.* **1988**, *65*, 1599−1600.

Bjorkqvist, B.; Hase, A. Separation and determination of intact glucosinolates in rapeseed by high-performance liquid chromatography. J. Chromat. **1988**, *435*, 501−507.

Boehringer Mannheim. Methods of biochemical analysis and food analysis. Boehringer Mannheim GmbH, Biochemica, 6800 Mannheim 31, West Germany, 1989.

Carlson, D. G.; Daxenbichler, M. E.; van Etten, C. H.; Tookey, H. L.; Williams, P. H. Glucosinolates in crucifer vegetables: Turnips and rutabagas. *J. Agric. Food Chem.* **1981**, *29*, 1235−1239.

Carlson, D. G.; Daxenbichler, M. E.; VanEtten, C. H.; Kwolek, W. F.; Williams, P. H. Glucosinolates in crucifer vegetables: Broccoli, Brussels sprouts, Cauliflower, Collards, Kale, Mustard greens, and Kohlrabi. *J. Am. Soc. Hortic. Sci.* **1987**, *112*, 173−178.

Daxenbichler, M. E.; VanEtten, C. H.; Williams, P. H. Glucosinolates and derived products in cruciferous vegetables. Analysis of 14 varieties of Chinese cabbage. *J. Agric. Food Chem.* **1979**, *27*, 34−37.

Fenwick, G. R. Rapeseed as an animal feedingstuff−The problems and analysis of glucosinolates. *J. Assoc. Public Analysts* **1984**, *22*, 117−130.

Fenwick, G. R.; Heaney, R. K.; Mullin, W. J. Glucosinolates and their breakdown products in food and feedingstuffs. *Crit. Rev. Food Sci. Nutr.* **1983a**, *18*, 123−206.

Fenwick, G. R.; Griffiths, N. M.; Heaney, R. K. Bitterness in Brussels sprouts (*Brassica oleracea* L. var. gemmifera): The role of glucosinolates and their breakdown products. *J. Sci. Food Agric.* **1983b**, *34*, 73−80.

Finnigan, T. J. A.; Lewis, M. J. Ethanolic extraction of commercial rapeseed meal in the production of protein concentrate of reduced glucosinolate content. *J. Sci. Food Agric.* **1988**, *45*, 155−163.

Griffiths, N. M.; Fenwick, G. R. Sensory assessment of glucosinolates (and their breakdown products) in cruciferous foods. In *Progress in Flavour Research*, Proceedings of the 4th Weurman Flavour Research Symposium, Dourdan, France, May 9−11, 1984; Elsevier Science Publishers BV: Amsterdam, 1984.

Hanley, A. B.; Heaney, R. K.; Fenwick, G. R. Improved isolation of glucobrassicin and other glucosinolates. *J. Sci. Food Agric.* **1988**, *34*, 869−873.

Hassan, F.; Rothnie, N. E.; Yeung, S. P.; Palmer, M. V. Enzyme linked immunosorbent assays for alkenyl glucosinolates. *J. Agric. Food Chem.* **1988**, *36*, 398−403.

Heaney, R. K.; Fenwick, G. R. Glucosinolates in Brassica vegetables. Analysis of 22 varieties of Brussels sprout (*Brassica oleracea* var. gemmifera). *J. Sci. Food Agric.* **1980a**, *31*, 785−793.

Heaney, R. K.; Fenwick, G. R. The glucosinolate content of Brassica vegetables. A chemotaxonomic approach to cultivar identification. *J. Sci. Food Agric.* **1980b**, *31*, 794−801.

Heaney, R. K.; Spinks, E. A.; Fenwick, G. R. The glucosinolate content of Brussels sprouts: Factors affecting their relative abundance. *Z. Pflanzenzuchtg.* **1983**, *91*, 219−226.

Hill, C. B.; Williams, P. H.; Carlson, D. G.; Tookey, H. L. Variation in glucosinolates in oriental *Brassica* vegetables. *J. Am. Soc. Hortic. Sci.* **1987**, *112*, 309-313.

Kuan, S. S.; Ngeh-Ngwainby, J.; Guilbault, G. G. Preliminary study on analytical methods for the determination of thioglucosides after enzymatic hydrolysis. *Anal. Lett.* **1986**, *19*, 887−899.

Lewis, J.; Fenwick, G. R. Glucosinolate content of *Brassica* vegetables: Analysis of twenty-four cultivars of calabrese (green sprouting broccoli, *Brassica oleracea* L. var. botrytis subvar. cymosa Lam.). *Food Chem.* **1987**, *25*, 259−268.

Lewis, J.; Fenwick, G. R. Glucosinolate content of *Brassica* vegetables-Chinese cabbages Pe-tsai (*Brassica pekinensis*) and Pak-choi (*Brassica chinensis*). *J. Sci. Food Agric.* **1988**, *45*, 379−386.

McGregor, D. I.; Mullin, W. J.; Fenwick, G. R. Review of analysis of glucosinolates: Analytical methodology for determining glucosinolate composition and content. *J. Assoc. Off. Anal. Chem.* **1983**, *66*, 825−849.

Palmieri, S.; Iori, R.; Leoni, O. Comparison of myrosinase activity assays. In *Glucosinolates in rapeseeds: Analytical aspects*; Wathelet, J. P., Ed.; Martinus Nijhoff Publishers: Dordrecht, The Netherlands, 1987; pp 14−25.

Peterka, S.; Fenwick, G. R. The use of flash chromatography for the isolation and purification of glucosinolates (mustard oil glycosides). *Fat Sci. Technol.* **1988**, *90*, 61−64.

Pocock, K.; Heaney, R. K.; Wilkinson, A. P.; Beaumont, J. E.; Vaughan, J. G.; Fenwick, G. R. Changes in myrosinase activity and isoenzyme pattern, glucosinolate content and the cytology of myrosin cells in the leaves of heads of three cultivars of English white cabbage. *J. Sci. Food Agric.* **1987**, *41*, 245−257.

Schnug, E.; Haneklaus, S. Theoretical principles for the indirect determination of the total glucosinolate content in rapeseed and meal quantifying the sulphur concentration via X-ray fluorescence (X-RF method). *J. Sci. Food Agric.* **1988**, *45*, 243−254.

Shasidi, F.; Gabon, J. E.; Rubin, L. J.; Naczk, M. Effect of methanol−ammonia−water treatment on the fate of glucosinolates. *J. Agric. Food Chem.* **1990**, *38*, 251−255.

Smith, D. B.; Parsons, D. G.; Starr, C. A simple and rapid method of quantitatively measuring the glucosinolate concentration of rapeseed. *J. Agric. Sci. Camb.* **1985**, *105*, 597−603.

Sones, K.; Heaney, R. K.; Fenwick, G. R. Glucosinolates in Brassica vegetables. Analysis of twenty-seven cauliflower cultivars (*Brassica oleracea* L. var. botrytus subvar. cauliflora DC). *J. Sci. Food Agric.* **1984**, *35*, 762−766.

Springett, M. B.; Adams, J. B. Identification of 1-cyano-2,3-epithiopropane in volatiles of a model system from Brussels sprouts (*Brassica oleracea* L var Bullata Subvar Gemmifera DC). *J. Sci. Food Agric.* **1988**, *46*, 211−219.

Springett, M. B.; Adams, J. B. Properties of Brussels sprouts thioglucosidase. *Food Chem.* **1989**, *33*, 173−186.

Thangstad, O. P.; Iversen, T.-H.; Slupphaug, G.; Bones, A. Immunocytochemical localization of myrosinase in *Brassica napus* L. *Planta* **1990**, *180*, 245−248.

Thies, W. Photometric determination of indolyl glucosinolates in seeds of rapeseed. *Fat Sci. Technol.* **1990**, *92*, 161−164.

Tholen, J. T.; Buzza, G.; McGregor, D. I.; Truscott, R. J. W. Measuring of the glucosinolate content in rapeseed using the trubluglu meter. *Plant Breed.* **1993**, *110*, 137−143.

Uda, Y.; Maeda, Y. Volatile constituents occurring in autolyzed leaves of three cruciferous vegetables. *Agric. Biol. Chem.* **1986**, *50*, 205−208.

Van Doorn, J. E.; Goormachtig, P. F. J.; de Haan, A. Influence of perchloric acid on ion-pair high-performance liquid chromatography of nucleotides. *J. Chromatogr.* **1989**, *496*, 441−449.

Van Doorn, J. E.; van der Kruk, G. C.; van Holst, G.-J.; Jongen, W. An assay for the determination of the total glucosinolate content in sugar containing samples of Brussels sprouts. *J. Agric. Food Chem.* **1997**, submitted for publication.

VanEtten, C. H.; Daxenbichler, M. E.; Williams, P. H.; Kwolek, W. F. Glucosinolates and derived products in cruciferous vegetables. Analysis of the edible part from twenty-two varieties of cabbage. *J. Agric. Food Chem.* **1976**, *24*, 452−455.

VanEtten, C. H.; Daxenbichler, M. E.; Tookey, H. L.; Kwolek, W. F.; Williams, P. H.; Yoder, O. C. Glucosinolates: Potential toxicants in cabbage cultivars. *J. Am. Soc. Hortic. Sci.* **1980**, *105*, 710−714.

Visentin, M.; Tava, A.; Iori, R.; Palmieri, S. Isolation and identification of trans-4-(methylthio)-3-butenyl glucosinolate from radish roots (*Raphanus sativus* L.). *J. Agric. Food Chem.* **1992**, *40*, 1687−1691.

Wilkinson, A. P.; Rhodes, M. J. C.; Fenwick, R. G. Myrosinase activity of cruciferous vegetables. *J. Sci. Food Agric.* **1984**, *35*, 543−552.

Yen, G.-C.; Wei, Q.-K. Myrosinase activity and total glucosinolate content of cruciferous vegetables, and some properties of cabbage myrosinase in Taiwan. *J. Sci. Food Agric.* **1993**, *61*, 471−475.

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