HARNESSING THE POWER OF CRUCIFEROUS VEGETABLES: DEVELOPING A BIOMARKER FOR *BRASSICA* VEGETABLE CONSUMPTION USING URINARY 3,3´-DIINDOLYLMETHANE

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HARNESSING THE POWER OF CRUCIFEROUS VEGETABLES: DEVELOPING A BIOMARKER FOR *BRASSICA* VEGETABLE CONSUMPTION USING URINARY 3,3´-DIINDOLYLMETHANE

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ABSTRACT

Background: Glucobrassicin, a predominant glucosinolate in *Brassica* vegetables, gives rise to indole-3-carbinol (I3C), a compound with potent anti-cancer effects in preclinical models. We previously showed that urinary measurement of 3,3´-diindolylmethane (DIM, the predominate metabolite of I3C), a potential exposure biomarker, can discriminate between human volunteers given high and low doses of glucobrassicin-containing *Brassica* vegetables. However, the quantitative relationship between glucobrassicin exposure and urinary DIM level is unclear. We hypothesized that a range of glucobrassicin exposure from *Brassica* vegetables is reflected in urinary DIM. We also hypothesized that this effect plateaus.

Methods: Forty-five subjects were randomly assigned to consume vegetables containing 1 of 7 discrete dose levels of glucobrassicin ranging from 25 to 500 μmol. The vegetables, a mixture of 'Jade Cross' brussels sprouts and 'Blue Dynasty' cabbage, were eaten once daily for two consecutive days. All urine was collected for 24 hours after each vegetable-eating session. Urinary DIM was measured using our published liquid chromatography-electrospray ionization-tandem mass spectrometry-selected reaction monitoring (LC/ESI-MS/MS-SRM) method.

Results: Urinary DIM excretion increased predictably with increasing glucobrassicin dose and plateaued between 200 and 300 μmol of glucobrassicin. Correlation between glucobrassicin dose and urinary DIM was strong and positive (R=0.82). The majority of DIM was excreted in the first 12 hours after vegetable consumption.

Conclusion: The positive and strong correlation between glucobrassicin dose and urinary DIM supports its use as a biomarker of glucobrassicin exposure and I3C uptake. Feeding

glucobrassicin beyond 200 μmol did not consistently lead to more urinary DIM. Evidence indicates that urinary DIM accurately reflects a dietary *Brassica* vegetable consumption based on glucobrassicin concentration.

Significance: Validating urinary DIM as a biomarker of gluocobrassicin exposure and an objective measure of *Brassica* vegetable consumption are critical for the design of future epidemiologic and chemoprevention studies. This biomarker will also be useful in future studies that seek to define biologically relevant doses for cancer prevention.

INTRODUCTION

The anti-cancer effect of cruciferous vegetables is widely thought to be mediated by their glucosinolates,[1] and epidemiologic evidence points to an association between high cruciferous vegetable consumption and decreased cancer risk,[2] but the association is inconsistent, likely due in large part to a lack of objective measures of phytonutrient exposure. Upon plant cell damage (i.e., chewing), inert glucosinolates are converted to indoles and isothiocyanates by myrosinase. Glucobrassicin, a predominant glucosinolate in common *Brassica* vegetables,[3,4] is hydrolyzed to indole-3-carbinol (I3C). In the acidic environment of the stomach, I3C readily undergoes acid condensation, primarily to 3,3´-diindolylmethane (DIM).[5-10] Both I3C and DIM possess strong anti-cancer effects in vitro and in-vivo.[11-13] I3C is rapidly hydrolyzed to DIM and other oligomers in vivo, and because of this, development of a blood-based biomarker is not realistic, nor have previous attempts been successful.[6,7,14] Alternatively, urine is a non-invasive and practical biospecimen ideal for use in large studies. We previously developed a novel method to quantify DIM in urine, and showed that consuming vegetables with divergent glucobrassicin concentrations was consistently reflected in urinary DIM levels.[15] To further define the utility of urinary DIM as a non-invasive biomarker of glucobrassicin exposure and I3C uptake from vegetables in humans, we conducted a clinical trial to determine the ability of urinary DIM to discriminate a wide range of glucobrassicin doses. We also sought to define the glucobrassicin dose at which this relationship plateaus, as observed with I3C and DIM administered as supplements.

MATERIALS AND METHODS

Study Design. Healthy, non-smoking, non-vegetarian adult subjects aged 18-60 years were recruited. Inclusion criteria required that subjects have normal kidney and liver function. Subjects taking H_2-blockers, proton pump inhibitors, or calcium carbonate regularly, and those recently treated with antibiotics were excluded. Questionnaires were administered to collect demographic information, and histories of tobacco, medication, and alcohol use. At enrollment, subjects were randomly assigned to one of 7 doses of glucobrassicin – 25, 50, 100, 200, 300, 400 or 500 μmol. Four subjects were recruited for each dose level. To determine inter-individual variation in urinary DIM between glucobrassicin doses, an additional 6 subjects were recruited for three dose levels – 50, 200 and 500 μmol. Eligible subjects refrained from cruciferous vegetable consumption for a minimum of 5 days prior to the intervention. Salads comprising fixed proportions of Brussels sprouts and cabbage to attain the desired glucobrassicin dose (see Table 1 in Supplemental Data) were freshly prepared with minimal chopping on the day of the study intervention. Subjects fasted 2 h before and 2 h after vegetable consumption and consumed the assigned dose of study vegetables once daily for 2 consecutive days at the study center as quickly as possible. A spot urine sample was collected prior to consuming the vegetables, and all urine was collected for 24 h following each vegetable-eating session, divided into the following periods: 0–2 h, 2–6 h, 6–12 h, and 12–24 h. This was done to inform whether briefer collection periods could be used in future studies. Urine volume was measured and aliquots stored at -20°C. Self-reported food diaries were kept throughout the study period and reviewed for obvious dietary intake of cruciferous

vegetables. The protocol and consent form were approved by the Institutional Review Board at the University of Minnesota. All subjects provided informed consent.

Analysis of Glucobrassicin Concentration in the Vegetables. 'Blue Dynasty' cabbage and 'Jade Cross' Brussels sprouts were grown and cultivated specifically for this study (see Supplemental Materials). Approximately 200 g, bulked from different heads of cabbage (n=4) and Brussels sprouts (n=4), were taken for determination of glucobrassicin concentration at one time point. Glucobrassicin concentration was analyzed using a published technique.[15]

Urine Sample Preparation. Urine samples were prepared using a previously published technique.[15] Analysts were blinded, but to minimize intra-individual variability all samples from a given subject were prepared together in a single set.

LC-ESI-MS/MS-SRM. Analysis was performed as previously published,[15] with slight modifications to increase sample throughput. Briefly, a TSQ Vantage instrument was used in addition to the TSQ Quantum Discovery Max instrument used previously. The TSQ Vantage was coupled to an Eskigent NanoLC Ultra (Eskigent Technologies) liquid chromatographic system; MS parameters on the TSQ Vantage were comparable to those previously published.[15] The chromatographic method was shortened to decrease analysis time. Initial conditions were 40% 10mM NH_4OAc and 60% methanol, percent methanol was then increased to 70% over 15 min and held for 1 min, then increased to 95% over 1 min and held for 2 min, the system was then returned to initial conditions over 1 min and

allowed to re-equilibrate for 10 min before injection of the next sample. Flow rate was held constant at 10 μL/min throughout the separation. The column was equipped with a KrudKatcher 0.5μ pre-filter (Phenomenex), which was exchanged after approximately 50 sample injections or when significant peak-broadening was observed. Typical retention times for DIM and [$^{2}H_{2}$]DIM on the Vantage system were 14.7 and 14.8 min respectively, and 16.6 and 16.5 on the Discovery. The detection limit of the assay was 0.4 pmol/mL.

Quality Control. This laboratory method for DIM has been previously validated.[15] However, considerable effort was put into to ensuring continued validity of the analysis. Each set included a minimum of two water blanks, spiked with [$_{2}H^{2}$]DIM to monitor recovery, as well as four positive controls consisting of urine samples confirmed to contain DIM, pooled from subjects who had consumed Brussels sprouts. Spiked control samples were included in select sets to confirm reproducibility at the lower and upper ends of the observed DIM concentration range in study samples. Briefly, baseline urine samples from 9 subjects, which had been confirmed to contain no measurable DIM, were pooled and then spiked to yield final DIM concentrations of 35 pmol/mL or 0.90 pmol/mL, or slightly over 2 times the detection limit of the assay. Samples with an apparent recovery below 5% were re-assayed.

Statistical Analysis. The primary objective of this study was to characterize the relationship between glucobrassicin dose and urinary DIM, specifically to identify the maximum dose where the urinary DIM 'response' levels off (plateau). The two 24 h DIM

measurements at each dose were averaged. Any DIM detected in the baseline spot urine sample prior to vegetable intake was subtracted from this value. The model used to determine the dose-response curve of glucobrassicin fed (dose) and urinary DIM (response) was based on the "median effect principle" of pharmacology[16] and expressed as a four parameter logistic equation:

$$\log(C/1-C) = \beta_1+\beta_2*(\log(\text{dose})), \quad \text{where } C=(\text{DIM}-\beta_3)/(\beta_4-\beta_3)$$

The four parameters estimated by this model are β_1 and β_2, the intercept and slope of the linear portion of the sigmoidal curve, and β_3 and β_4, the minimum and maximum levels, respectively, of urinary DIM where the logistic curve flattens out at lower and higher doses. These parameters are estimated using the non-linear (NLIN) procedure in SAS version 9.3 (SAS Institute Inc., USA). The dose that produces a 50% response (ED50) is calculated by the estimates of the intercept and slope: ED50= exp[(- 1)(intercept/slope)]. The minimum, maximum DIM and EC50 are reported with their 95% confidence intervals. The minimum and maximum estimates were entered into the above equation to calculate (C/1-C), which results in a simple linear expression in the logarithmic scale and, therefore the correlation (R) between glucobrassicin dose and urinary DIM was calculated.

All means are reported with their SE and/or 95% confidence intervals unless otherwise noted.

RESULTS

Subject Characteristics and Study Compliance: Nineteen males and 26 females ranging in age from 19-56 years (mean 31.5 ± 1.5 years) completed the study. One subject at the 50 μmol dose dropped out due to issues unrelated to the study and was not replaced. All other subjects are included in the data analysis. Thirty-five (78%) were Caucasian, 5 were Asian (11%), 2 were African-American (4%) and 3 (7%) reported more than one race. At the 500 μmol dose level, two subjects could not finish due to the taste of the raw Brussels sprouts and were reassigned to the 50 μmol dose after a minimum 1 week washout period. Additionally, subject 40 at the 500 μmol dose finished only 162.1 g (66.8%) of the Brussels sprouts on day 1 and 210.5 g (86.7%) on day 2. Subject 43 at the 500 μmol dose finished 90.5 g (37.3%) on day 1 and 188.1 g (77.5%) on day 2. Subject 36 at the 500 μmol dose finished 128.1 g (52.3%) on day 1 and 184.8 g (76.1%) on day 2. At the 200 μmol dose level, one subject finished only 52% of the vegetables on day 1 and 75% of the vegetables on day 2 due to taste intolerance. Since these salads were a mix of cabbage and Brussels sprouts, it is not possible to determine the exact amount of each consumed. Of the 352 total urine collection periods (8 collection periods per subject x 44 subjects), only 4 partial urine voids were missed. Subject 39 missed one void on day 2 during the 2-6 h collection period. Subject 44 missed collecting 2 voids on day 2 during the 6-12 h urine collection. Urine collection compliance was based on self-report.

Glucobrassicin concentration: The cabbage (n=4 samples) contained 33.5 ± 4.0 μmol

per 100 grams food weight. The Brussels sprouts (n=4 samples) contained 206.0 ± 12.9 μmol per 100 grams food weight. Both are consistent with prior results.[15]

Quality Control: Inter-day precision was 8.7% and intra-day precision was 3.9% (n=68 across 12 sets). Measured DIM concentration (N=14 samples, split between 5 sets) in the 35 pmol/mL spiked samples was 33.3 ± 2.5 (SD) pmol DIM/mL (CV 7.5%) and in the 0.9 pmol/mL spiked samples was 0.87 ± 0.08 (SD) pmol DIM/mL (CV 9.2%).

Dose-response between glucobrassicin exposure and urinary DIM excretion: Baseline urinary DIM levels, 24 hour concentrations after consumption of the study vegtables, and percent excreted in the first 12 hours of the 24 h urine collection period are shown in Table 1. As shown in Figure 1, urinary DIM excretion plateaued between glucobrassicin doses of 200 and 300 μmol. The correlation (R) between measured DIM and predicted DIM across all subjects was 0.82, indicating a strong correlation. Excluding the three subjects at the 500 μmol dose who could not finish the Brussels sprouts due to taste intolerance did not change the correlation (R=0.82). We then analyzed the intraclass correlation (ICC), or how similar the two 24 h urinary DIM levels from an individual compare to the similarity in other individuals. The ICC at the three expanded dose level cohorts is shown in Table 2. At 50 μmol, variability in 24 h urinary DIM levels appears to stem from both within an individual and between indivduals. At the 200 and 500 μmol dose levels, most of the variability is coming from between individuals rather than within an individual. We next examined the kinestics of urinary DIM excretion. On average, 95.1 ± 1.2% (95% CI 92.7, 97.5) on day 1 and 96.1 ± 0.8%

(95% CI 94.5, 97.7) on day 2 of the total DIM excreted was excreted in the first 12 hours after vegetable consumption.

DISCUSSION

We showed, for the first time in humans, that urinary DIM accurately reflects exposure to a wide range of glucobrassicin concentrations in vegetables. Ours is also the first study to demonstrate that urinary DIM excretion plateaus after eating vegetables. Similar pharmacokinetic profiles were observed when I3C or DIM was administered to humans as supplements.[14,17] Interestingly, the dose of glucobrassicin at which the plateau occurs after vegetable consumption is ~5000-fold less than when I3C or DIM are taken as supplements, suggestive of a vast difference in bioavailability and absorption. DIM, the predominant acid condensation heterodimer of I3C, has poor bioavailability as a supplement due to its poor water solubility, although an absorption-enhanced form has become available.[18] The factors that influence the relative bioavailability of I3C or DIM after food consumption and the major route of their excretion in humans remain largely uncharacterized, although gastric pH influences the relative abundance of oligomers derived from I3C, whereby more acidic conditions favor the formation of higher order oligomers.[5] For this reason, participants in our study were asked to fast and were not on medications that might affect gastric pH. However, our approach of using urinary DIM to quantify I3C uptake potentially bypasses this issue and can mitigate other sources of variation in glucobrassicin/I3C exposure (despite individuals eating identical amounts of glucobrassicin) such as cultivar, growing conditions, and preparation method.[3,19] Furthermore, DIM appears to be a good surrogate, as I3C itself rapidly hydrolyzed and

therefore quickly undetectable in vivo, whereas DIM is quite stable.[10,14] Importantly, our data support the notion that the cancer-preventive properties that might be derived from cruciferous vegetable consumption may not require a large quantity of vegetables nor high-dose supplements, which certainly has practical implications.[20] The optimal dose, duration, and frequency of vegetable consumption remain to be worked out.

Our results indicate that DIM is excreted in the urine rapidly after glucobrassicin consumption, consistent with pharmacokinetic studies done after single- and multiple-dose I3C[14] and single-dose DIM,[17] and consistent with our prior study.[15] This implies that daily or more frequent dosing may be necessary to maximize phytonutrient exposure. Additionally, our data suggest that a full 24 h urine collection may not be necessary to capture the magnitude of I3C exposure, and that a 12 h urine collection will suffice. This will improve compliance and feasibility of future studies.

Consistent with prior studies,[21] we observed inter-individual variability in 24 h urinary DIM levels at each glucobrassicin dose level. However, the correlation between measured and predicted DIM remained very strong. Furthermore, at moderate to higher doses (200 and 500 μmol glucobrassicin), we did not see significant variability between the two 24 h urinary DIM measurements from an individual subject. In other words, the majority of the variability within a dose level occurs between subjects, not within a subject, therefore, a single urine collection after consuming a known dose of glucobrassicin is reliable. To this end, it may be that this inter-individual variability reflects the relative benefit an individual derives from consuming glucobrassicin from

vegetables. This has never been studied in humans. This data also supports urinary DIM's utility as a biomarker, responsive to sources of variation in glucobrassicin exposure, I3C uptake and DIM metabolism. We will explore this hypothesis in future studies.

In conclusion, the amount of DIM excreted in the urine correlates with the amount of glucobrassicin consumed from vegetables, making it an easily accessible, non-invasive biomarker of glucobrassicin exposure and I3C uptake. Feeding glucobrassicin beyond 200 μmol, or ~100 g of raw Brussels sprouts, did not consistently lead to more urinary DIM. This represents a first step in defining a biologically relevant dose of *Brassica* vegetable consumption based on glucobrassicin. Our work sets the stage for objectively quantifying I3C uptake from the diet in epidemiological studies, overcoming major limitations of observational studies. Furthermore, we can now correlate I3C uptake in glucobrassicin-based vegetable feeding interventions with outcomes in chemoprevention studies.

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TABLE 1: Baseline urinary DIM, 24 h urinary DIM, and % excreted in 12 hours

Subject #	glucobrassicin dose (μmol)	Baseline DIM (pmol/mL)	24 h DIM (pmol/mL)		Mean 24 h DIM ± SE (pmol/mL)	DIM excreted in 12 hours (%)	
			Day 1	Day 2		Day 1	Day 2
1	25	<LOD	7.91	1.84	4.88 ± 2.15	100	100
2		ND	7.27	4.70	5.99 ± 0.91	91.8	100
3		<LOD	28.12	49.05	38.59 ± 7.40	92.2	100
4		1.24	8.83	3.83	6.33 ± 1.77	93.2	100
5	50	ND	3.19	12.73	7.96 ± 3.37	100	100
6		<LOD	2.00	2.71	2.36 ± 0.25	60.0	81.5
7*		<LOD	11.93[¶]	10.64	11.29 ± 0.46	95.0	95.3
8		ND	14.73	15.60	15.17 ± 0.31	100	100
9*		<LOD	54.61	15.06	34.84 ± 13.98	98.9	100
10		0.82	13.76	22.12[∞]	17.94 ± 2.96	81.2	84.2
11		0.82	5.99	43.27	24.63 ± 13.18	88.3	98.6
12		ND	5.69	2.54	4.12 ± 1.11	100	100
13		<LOD	49.16	47.23	48.20 ± 0.68	97.6	96.8
14	100	<LOD	175.08	171.26	173.17 ± 1.35	99.5	98.5
15		ND	121.72	126.76	124.24 ± 1.78	98.8	95.7
16		2.45	35.35	66.15	50.75 ± 10.89	98.3	96.4
17		<LOD	105.68	47.23	76.46 ± 20.67	96.9	100
18	200	2.71	339.56	58.41	198.99 ± 99.40	99.2	91.3
19		<LOD	300.04	128.75	214.40 ± 60.56	96.5	91.1
20		ND	1148.90	1061.42	1105.16 ± 30.93	98.7	95.6
21		<LOD	49.08	26.48	37.78 ± 7.99	97.8	96.6
22		<LOD	125.99	193.10	159.55 ± 23.73	96.2	99.4
23		<LOD	106.50	39.03	72.77 ± 23.85	98.2	84.7
24		1.30	347.27	225.85	286.56 ± 42.93	94.8	100
25		<LOD	508.88	614.47	561.68 ± 37.33	97.8	100
26		ND	502.44	335.45	418.95 ± 59.04	93.1	100
27		<LOD	16.70	24.72	20.71 ± 2.84	95.8	93.1
28	300	<LOD	517.24	553.70	535.47 ± 12.89	99.7	99.6
29		<LOD	91.15	133.98	112.57 ± 15.14	97.5	97.9
30		<LOD	64.90[§]	105.90	85.40 ± 14.50	96.1	95.5
31		<LOD	456.83	106.09	281.46 ± 124.01	99.6	97.2
32	400	ND	738.15	957.16	847.66 ± 77.43	95.8	91.5
33		<LOD	625.40	481.79	553.60 ± 50.77	98.4	98.5
34		<LOD	76.27	158.80	117.54 ± 29.18	95.0	97.5
35		<LOD	151.16[¶]	406.10	278.63 ± 90.13	73.6	91.2
36	500	0.74	157.19	133.52	145.36 ± 8.37	82.3	76.3
37		<LOD	301.79^	786.77	544.28 ± 171.47	99.1	99.3
38		<LOD	352.00^	314.48^	333.24 ± 13.27	97.9	97.3
39		ND	2263.64	2096.57	2180.11 ± 59.07	99.5	99.0
40		5.72	958.91	2044.33	1501.62 ± 383.75	96.2	92.1
41		ND	1264.93	108.86[#]	686.90 ± 408.73	100	93.7
42		<LOD	591.33^	911.23^	751.28 ± 113.10	99.4	99.4
43		<LOD	108.66	74.88	91.77 ± 11.94	97.7	95.3
44		<LOD	122.43^	347.78^	235.11 ± 79.67	100	100
45		<LOD	180.24	108.48[†]	144.36 ± 25.37	93.1	85.7

* Originally randomized to 500 μmol dose level but reassigned to 50 μmol dose level due to intolerance

¶ Missed one void during the 12-24 hour collection period
∞ Missed one void during 6-12 hour collection period
§ One void from 6-12 hour collection period inadvertently collected in the container allocated to the 12-24 hour
collection period
^ Did not eat all of the assigned vegetables
# Missed one void during 2-6 hour collection period
† Missed 2 voids during the 6-12 hour collection period
LOD Level of detection
ND Not detectable

TABLE 2: Intraclass correlation (ICC) of urinary DIM at 3 glucobrassicin dose levels

Glucobrassicin dose (μmol)	ICC
50	0.41
200	0.94
500	0.71

ICC here is a measure of how similar urinary DIM levels from the two 24 h collections within an individual compare to the similarity between subjects at the same dose level.

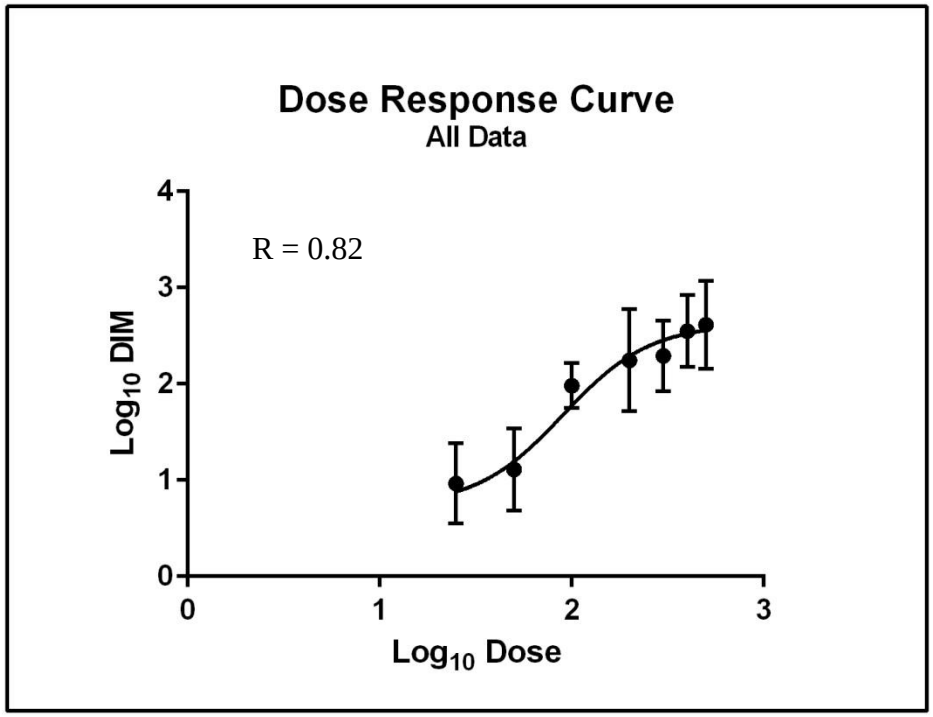

Figure 1: Dose curve between glucobrassicin dose (25-500 μmol) and urinary DIM. Bars represent standard error. Glucobrassicin dose ranged from 25 μmol to 500 μmol. Estimated parameters in the original scale (95% CI): Maximum DIM 421.5 pmol/mL (154.7, 1148.4), minimum DIM 5.4 pmol/mL (0.7, 44.3), EC50 90.2 μmol (29.1, 151.3).

SUPPLEMENTAL MATERIALS

Composition of cabbage and Brussels sprout salads

Glucobrassicin dose	Cabbage			Brussels sprouts		
	Qty (g)	Proportion of dose	μmol	Qty (g)	Proportion of dose	μmol
25	74.63	100%	25	0	0%	0
50	119.40	80%	40	4.85	20%	10
100	149.25	50%	50	24.27	50%	50
200	179.10	30%	60	67.96	70%	140
300	134.33	15%	45	123.79	85%	255
400	119.40	10%	40	174.76	90%	360
500	0	0%	0	242.72	100%	500

Table 1: Proportion of cabbage and Brussels sprouts to attain the desired dose of glucobrassicin. Cabbage: 33.5 ± 4.0 μmol/100 g food weight. Brussels sprouts: 206.0 ± 12.9 μmol/100 g food weight.

Growing Conditions of 'Jade Cross' Brussels Sprouts and 'Blue Dynasty

'Jade Cross' Brussels sprouts and 'Blue Dynasty' cabbage (Jordan Seeds, Woodbury, MN) were seeded into 48-count cell (53 × 27 cm) trays containing moist soilless seeding media (Sunshine SB-300 Universal; Sun-Gro Horticulture, Bellevue, WA) on 23 May. Seedlings were grown in the Plant Growth Facilities on the University of Minnesota St. Paul campus until 5 July. At that time, transplants were hardened off at the Southern Research and Outreach Center in Waseca, MN. Transplants were planted 46 cm apart on raised beds 0.7 m wide and 1.5 m apart in Waseca on 19 July. The soil was Nicollet-Webster clay loam, fertilized with 168 kg ha^{-1} nitrogen (urea) before the raised beds were made. Plants were watered as needed with drip irrigation throughout the season. Cabbage was harvested on 4 October, placed in ventilated plastic bags, and stored in a cooler at ~3ºC with humidification. When the air temperature dropped below freezing, a spun polypropylene row cover was applied to the Brussels sprouts remaining in the field. Brussels sprouts stalks were cut on 30 October, and the harvested sprouts (U.S. No. 1 & 2 sprouts, USDA, 1997) were stored under the same conditions as cabbage.