

1 **Kale Carotenoids Remain Stable while Flavor Compounds**  
 2 **Respond to Changes in Sulfur Fertility†**

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 14 Dietary intake of certain carotenoids has been associated with a reduced risk of disease. Kale (*Brassica*  
 15 *oleracea* L. Acephala Group) has the highest levels of carotenoids lutein and  $\beta$ -carotene, and is an  
 16 excellent source of minerals among the green leafy vegetable crops. However, *Brassica* vegetables  
 17 contain glucosinolate (GS) and S-methylcysteine sulfoxide (MCSO). While these sulfur compounds  
 18 have medicinal value, they are also responsible for the bitter, acrid flavors that are often regarded as  
 19 objectionable by consumers. Therefore, the objectives of this study were to investigate the influence  
 20 of increased S fertility levels on (1) elemental accumulation, (2) GS and MCSO production, and (3)  
 21 the accumulation patterns of carotenoid pigments in the leaves of three kale cultivars. Winterbor,  
 22 Redbor, and Toscano kale were greenhouse-grown using nutrient solution culture with S treatment  
 23 concentrations of 4, 8, 16, 32, and 64 mg of S/L. Decreasing S fertility decreased S leaf content, but  
 24 increased the levels of Mg and Ca accumulation, two important minerals for human health. Levels of  
 25 GS and MCSO decreased in response to a decreasing S level in nutrient solution. However,  
 26 accumulation of lutein and  $\beta$ -carotene was unaffected by S treatment. Lowering the S fertility in the  
 27 production of kale should decrease the levels of negative flavors associated with high levels of GS  
 28 and MCSO without affecting carotenoid pigment levels. Understanding the combined impact of fertility  
 29 on flavor compounds and carotenoid pigments may help improve consumer acceptance of  
 30 phytonutritionally enhanced vegetable crops.  
 31

32 **KEYWORDS:** *Brassica oleracea*; glucosinolates; S-methyl-L-cysteine sulfoxide; macronutrients; micro-  
 33 nutrients; lutein;  $\beta$ -carotene; HPLC

34 **INTRODUCTION**

35 Foods have long been considered beneficial for human health  
 36 maintenance. Among those thought to have high nutritional and  
 37 medicinal value are the *Brassica* vegetables, which contain  
 38 essential dietary minerals and sulfur and carotenoid compounds.  
 39 Understanding how these compounds are metabolized in plants

and how they can be manipulated to enhance their medicinal 40  
 value has become increasingly important. 41

In plants, sulfur (S) is actively transported into roots as sulfate 42  
 ( $\text{SO}_4^{2-}$ ) and translocated to shoots and leaves. Sulfate can be 43  
 stored in cellular vacuoles, but reduction to  $\text{S}^{2-}$  is required prior 44  
 to incorporation into cysteine (1). Many plant species also 45  
 incorporate S into a wide range of secondary compounds 46  
 responsible for characteristic odors and flavors. S deficiencies 47  
 can appear as mild chlorosis and reductions in the rates of plant 48  
 growth. Conversely, some plant species have been shown to be 49

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50 comparatively insensitive to high  $\text{SO}_4^{2-}$  concentrations in the  
51 growing environment (2).

52 Glucosinolates [ $\beta$ -thioglucoside *N*-hydroxysulfates (GS)] and  
53 *S*-methylcysteine sulfoxide (MCSO) are secondary S-containing  
54 compounds present in *Brassica oleracea* L. (3). Glucosinolates  
55 and MCSO have no identifiable primary function in plants, but  
56 have been theorized to protect against predation and pathogens,  
57 as well as to act as S storage reserves (4). Following cellular  
58 disruption, GS and MCSO are enzymatically decomposed to  
59 produce compounds responsible for the characteristic flavor of  
60 *Brassica*. Breakdown products of GS have also been shown to  
61 possess anticarcinogenic activity and may be useful as chemo-  
62 preventative agents in humans (3).

63 More than 100 different GS compounds have been identified  
64 in plants, but only approximately 12 are found in *Brassica* (3).  
65 When GS compounds are decomposed by the enzyme myro-  
66 sinase ( $\beta$ -thioglucoside glucohydrolase, EC 3.2.3.1), glucose,  
67 bisulfate, and aglucones, which can then fragment or rearrange  
68 to form mixtures of volatile and nonvolatile compounds,  
69 including isothiocyanates, thiocyanates, and nitriles, are pro-  
70 duced (5, 6). Myrosinase and GS compounds are differentially  
71 sequestered in plant cells; however, upon tissue disruption during  
72 insect feeding, harvesting, processing, food preparation, mas-  
73 tication, or digestion, they are brought into contact, and  
74 decomposition occurs (7). The identity of the side chains of  
75 GS, which include aliphatic, aromatic, or heteroaromatic groups,  
76 determines the nature of the isothiocyanate, thiocyanate, or  
77 nitrile formed. Isothiocyanates are a group of hot and bitter  
78 compounds commonly termed "mustard oils" (8).

79 Carotenoids are  $\text{C}_{40}$  isoprenoid polyene secondary plant  
80 compounds that form lipid soluble yellow, orange, and red  
81 pigments. Examples of carotenoids include the oxygenated  
82 xanthophyll lutein [(3*R*,3'*R*,6'*R*)- $\beta$ , $\epsilon$ -carotene-3,3'-diol] and the  
83 hydrocarbon carotene  $\beta$ -carotene ( $\beta$ , $\beta$ -carotene; 9). Carotenoids  
84 span the thylakoid membranes of chlorophyll complexes and  
85 function in accessory roles of light harvesting, photoprotection,  
86 and structural stabilization (10, 11). Carotenoid pigments protect  
87 photosynthetic structures by quenching excited triplet chloro-  
88 phyll ( $^3\text{Chl}$ ) to dissipate excess energy (12) and binding singlet  
89 oxygen ( $^1\text{O}_2$ ) to inhibit oxidative damage (10, 11). The  
90 nutritional and medicinal importance of the dietary carotenoids  
91 is being established (13, 14). Plants are the primary sources of  
92 carotenoids in the diet, and leafy members of the Cruciferae,  
93 including subspecies of *B. oleracea* L., contain abundant  
94 amounts relative to other vegetables (15). Carotenoids exhibit  
95 antioxidant and anticarcinogenic activity (13, 14). Dietary intake  
96 of lutein,  $\beta$ -carotene, and other carotenoids has been associated  
97 with a reduced risk of lung cancer and chronic eye diseases,  
98 including cataract and age-related macular degeneration (16, 17).  
99 Studies have indicated that consumption of a variety of  
100 vegetables providing a mixture of carotenoids was more strongly  
101 associated with disease reduction than individual carotenoid  
102 supplements (16). Although these associative epidemiological  
103 relationships indicate carotenoids may serve photoprotective and  
104 antioxidant functions in humans, direct evidence of these actions  
105 is still lacking (18, 19).

106 Green leafy vegetables are rich in dietary carotenoids, and  
107 kale (*B. oleracea* L. Acephala Group) ranks the highest among  
108 all vegetable crops for reported lutein and  $\beta$ -carotene content  
109 (20). The *Brassica* vegetables are also good dietary sources of  
110 Ca and Mg (21). Glucosinolates and MCSO are S-containing  
111 compounds in *B. oleracea* responsible for flavor and potential  
112 health benefits. However, because these S compounds can  
113 impart negative flavor attributes at high tissue concentrations,

114 understanding how their levels can be lowered is important for  
115 consumer preference and fresh consumption. Therefore, the goal  
116 of this study was to investigate the influence of different S  
117 fertility levels on (1) mineral accumulation, (2) GS and MCSO  
118 production, and (3) the accumulation patterns of carotenoid  
119 pigments in the leaf tissues of three kale cultivars. Three kale  
120 cultivars were evaluated to assess the genetic variability  
121 previously reported for GS and carotenoid accumulation (22,  
122 23).

## MATERIALS AND METHODS

123  
124 **Plant Culture.** On January 31, 2002, three kale cultivars [Winterbor,  
125 Redbor, and Toscano (Johnny's Selected Seed, Albion, ME)] were  
126 seeded into rockwool cubes (Grodan A/S, Dk-2640 Hedehusene,  
127 Denmark). The medium was supplied with bottom heat (23 °C) and  
128 grown in a greenhouse (22 °C during the day and 14 °C at night) for  
129 2 weeks under natural photoperiods (latitude, 43° 09' N). Nutrients  
130 were applied as needed using 200 mg/L Peter's 20N-6.9P-16.6K water-  
131 soluble fertilizer (Grace-Sierra, Milpitas, CA).

132 On February 15, 2002, plants were transferred to 37.9 L containers  
133 (Rubbermaid Inc., Wooster, OH) having 30 L of a half-strength  
134 modified Hoagland's nutrient solution (24). Five plants of each of the  
135 three kale cultivars were placed into 2.22 cm holes with 10.6 cm  $\times$   
136 9.5 cm spacing on each container lid. Elemental concentrations of the  
137 nutrient solutions were as follows: 434.0 mg/L  $\text{NO}_3\text{-N}$ , 9.0 mg/L  $\text{NH}_4\text{-N}$ ,  
138 15.3 mg/L P, 117.1 mg/L K, 80.2 mg/L Ca, 24.6 mg/L Mg, 0.5  
139 mg/L Fe, 0.25 mg/L B, 0.005 mg/L Mo, 0.01 mg/L Cu, 0.25 mg/L  
140 Mn, and 0.025 mg/L Zn. Plants were grown under increasing S  
141 treatment concentrations at 4, 8, 16, 32, and 64 mg of S/L supplied as  
142  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ . Sulfur was partially supplied as  $\text{Na}_2\text{SO}_4$  during the  
143 treatment with 64 mg of S/L, and Mg was partially supplied as  $\text{MgCl}_2$   
144 during the treatments with 4, 8, and 16 mg of S/L to maintain a level  
145 of 24.6 mg of Mg/L across all the treatments. Solutions were aerated  
146 with an air blower (model VB-007S, Sweetwater, Ft. Collins, CO)  
147 connected to air stones. The experimental design was a split plot, with  
148 S treatment as the main plot and kale cultivar as the subplot. Each  
149 treatment consisted of five plants per cultivar and was replicated four  
150 times. Each solution was replaced every 2 weeks throughout the  
151 experiment to refresh the solution to the initial nutrient concentrations.

152 Plants were harvested on March 29, 2002. Once they were harvested,  
153 shoot and root tissue were separated, and shoot tissue from five plants  
154 in each treatment/cultivar combination was bulked and weighed for  
155 fresh mass accumulation. Plant tissues were washed with soap (Aquet,  
156 Bel-art Products, Pequannock, NJ), rinsed, and blotted dry with paper  
157 towels. One shoot tissue group was dried at 45 °C for no less than 72  
158 h, at which time the dry weight was calculated. One shoot tissue group  
159 was placed in a -80 °C freezer prior to lyophilization. Tissues were  
160 lyophilized for 48 h (model 6 L FreeZone, LabConCo, Kansas City,  
161 MO) prior to extraction.

162 **Elemental Determination.** Dried leaves were ground so they would  
163 pass through a 0.5 mm screen in a sample mill grinder (model 1093,  
164 Cyclotec-Tector, Höganäs, Sweden). A 0.3 g tissue sample was  
165 combined with 10 mL of concentrated nitric acid (70%  $\text{HNO}_3$ ) and  
166 digested in a microwave-accelerated reaction system (MARS5, CEM  
167 Corp., Matthews, NC). Digestion solutions were allowed to cool to  
168 room temperature (~26 °C) and adjusted to a final volume of 40 mL  
169 with deionized water. Elemental concentrations were determined by  
170 inductively coupled argon plasma atomic emission spectrometry (ICP-  
171 AES; model Vista AX, Varian, Inc., Palo Alto, CA).

172 **Glucosinolate and Methylcysteine Sulfoxide Determination.** *Glucosinolates.* (1) *Tissue Extraction.* Glucoiberin (3-methylsulfanylpropyl),  
173 sinigrin [prop-2-enyl(allyl)], glucobrassicin (3-indolylmethyl), neoglu-  
174 cobrassicin (1-methoxy-3-indolylmethyl), 4-methoxyglucobrassicin (4-  
175 methoxy-3-indolylmethyl), and 4-hydroxyglucobrassicin (4-hydroxy-  
176 3-indolylmethyl) were extracted from freeze-dried kale leaf tissue. For  
177 GS analysis, 0.1 mg samples were combined with 1 mL of benzyl GS  
178 solution (1 mM) as an internal standard, 2.0 mL of methanol, and 0.3  
179 mL of barium-lead acetate (0.6 M) in a 16 mm  $\times$  100 mm culture  
180 tube and shaken at 60 rpm for 1 h. Each tube was then centrifuged at  
181

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182 2000g<sub>n</sub> for 10 min. A 0.5 mL aliquot of supernatant was then added to  
183 a 1 mL column containing 0.3 mL of DEAE-Sephadex A-25. The  
184 sample was desulfated by the procedure of Raney and McGregor (25).

185 (2) *High-Performance Liquid Chromatography (HPLC) Analysis.*  
186 Extracted desulfoglucosinolates were separated with a Hewlett-Packard  
187 (Palo Alto, CA) HPLC system using a C-18 ODS reverse-phase column  
188 [250 mm × 4.6 mm (inside diameter), 5 μm] and a UV detector at a  
189 wavelength of 230 nm. The column temperature was set at 35 °C. A  
190 flow rate of 1.5 mL/min was used. For 1 min, the solvent was 100%  
191 water. This was followed by a 15 min linear gradient to 75% water  
192 and 25% acetonitrile. Solvent levels were then held constant for 5 min,  
193 and over the final 5 min, a linear gradient to 100% water was used.  
194 Desulfoglucosinolates were identified by comparison with retention  
195 times of authentic standards or previously reported results (22, 26).

196 *Methylcysteine Sulfoxide. (1) Tissue Extraction.* Ground sample leaf  
197 tissue was redried at 65 °C in a forced air oven (Linberg Blue, Asheville,  
198 NC). Methylcysteine sulfoxide (MCSO) was extracted from 0.2 g of  
199 dried tissue by adding 30 mL of a 12:5:3 methanol/chloroform/water  
200 mixture (MCW) and allowing it to incubate overnight at -20 °C (27).  
201 Sixty milliliters of chloroform was then added to the MCW extract,  
202 and the mixture was allowed to separate in a separatory funnel. A 2.5  
203 mL subsample of the polar fraction was dried using forced ambient air  
204 (Evap-O-Rac, Cole Parmer, Vernon Hills, IL). The dried sample was  
205 rehydrated in 1 mL of deionized and distilled water, and a 0.25 mL  
206 aliquot was taken and combined with 0.5 mL of an internal standard,  
207 (±)-ethylcysteine sulfoxide (ECSO) (1 mg/mL), prepared using a  
208 modification of the method of Lancaster and Kelly (28). The sample  
209 solution was then dried using forced ambient air and redissolved with  
210 1 mL of an aqueous hydrochloric acid [HCl (pH 2.5)] solution prior to  
211 HPLC analysis.

212 (2) *HPLC Analysis.* Sample MCSO concentrations were determined  
213 using the method of Edwards et al. (29). A Waters 2690 series HPLC  
214 unit with a photodiode array detector (PDA) (model 996, Waters Corp.,  
215 Milford, MA) was used for sample separation. Fifty microliters of the  
216 sample was injected into two C18 4.6 mm × 250 mm, 5 μm [ODS(2)  
217 SphereClone, Phenomenex] columns with one 4 mm × 3 mm C18  
218 guard column insert (Security Guard, Phenomenex). A dilute HCl (pH  
219 2.5) isocratic eluent was used with a flow rate of 0.9 mL/min. The  
220 eluent was filtered with 0.45 μm nylon filters (Micron Separations,  
221 Inc., Westboro, MA), and MCSO was detected at 210.2 nm. Peak  
222 assignment was performed by comparing retention times and line  
223 spectra obtained from PDA with an MCSO standard prepared according  
224 to the method of Lancaster and Kelly (28). Quantification was achieved  
225 by comparing the relative areas of the MCSO peak with those of the  
226 internal standard (ECSO) using Millennium Chromatography Software  
227 (Waters Corp.).

228 *Carotenoid and Pigment Determination. Tissue Extraction.* Freeze-  
229 dried tissues were combined with ~50 g of dry ice in a household  
230 food chopper (Handy Chopper Plus, Black & Decker, Towson, MD).  
231 Macerated tissues were placed in 20 mL scintillation vials, and CO<sub>2</sub>  
232 gas was vented prior to storage at -20 °C. Carotenoids and pigments  
233 were extracted and separated according to the method of Beecher and  
234 Howard (U.S. Department of Agriculture Food Composition Laboratory,  
235 Beltsville, MD), which is based on the method of Khachik et al. (30).  
236 A 0.10 g subsample was rehydrated with 0.8 mL of ddH<sub>2</sub>O at 40 °C  
237 for 20 min. After incubation, 0.8 mL of the internal standard ethyl  
238 β-8-apocarotenoate (Sigma Chemical Co., St. Louis, MO) and 2.5 mL  
239 of tetrahydrofuran (THF) stabilized with 25 ppm 2,6-di-*tert*-butyl-4-  
240 methoxyphenol (BHT) were added. The sample was homogenized in  
241 a Potter-Elvehjem (Kontes, Vineland, NJ) tissue grinding tube using  
242 ~25 insertions with a pestle attached to a drill press (Craftsman 15 in.  
243 drill press, Sears, Roebuck and Co., Hoffman Estates, IL) set at 540  
244 rpm. During homogenation, the tube was immersed in ice to dissipate  
245 heat. The tube was then placed in a clinical centrifuge for 3 min at  
246 500g<sub>n</sub>. The supernatant was removed, and the sample pellet was  
247 resuspended in 2.0 mL of THF and homogenized again with the same  
248 extraction technique. The extraction procedure was repeated two more  
249 times until the supernatant was colorless. The volumes of the combined  
250 supernatants were reduced to 0.5 mL using nitrogen (model N-EVAP  
251 111, Organomation Inc., Berlin, MA) at 40 °C, and 2.5 mL of MeOH  
252 and 2.0 mL of THF were added to the sample prior to HPLC analysis.

253 *HPLC Analysis.* An Agilent 1100 series HPLC unit with a PDA  
254 detector (Agilent Technologies) was used for sample separation. All  
255 samples were analyzed for carotenoid and chlorophyll compounds using  
256 a Vydac RP-18 5.0 μm, 250 mm × 4.6 mm column (model 201TP54,  
257 Phenomenex, Torrance, CA) fitted with a 4 mm × 3.0 mm, 7.0 μm  
258 guard column (RP-18, Phenomenex). The column was maintained at  
259 16 °C using a thermostated column compartment. Eluents were (A)  
260 75% acetonitrile, 20% methanol, 5% hexane, 0.05% BHT, and 0.013%  
261 triethylamine (TEA) in water (v/v) and (B) 50% acetonitrile, 25% THF,  
262 25% hexane, and 0.013% TEA in water (v/v). The flow rate was 0.7  
263 mL/min, and the gradient is as follows: 100% A for 30 min, 50% A  
264 and 50% B for 2 min, 100% B for 2 min, and 50% A and 50% B for  
265 2 min. The eluent composition was returned to 100% A, and the column  
266 was equilibrated for 10 min prior to the next injection. Eluted carotenoid  
267 and chlorophyll compounds from a 20 μL injection were detected at  
268 452, 652, and 665 nm, and data were collected, recorded, and integrated  
269 using 1100 HPLC ChemStation Software (Agilent Technologies). Peak  
270 assignment was performed by comparing retention times and line  
271 spectra obtained from the PDA with those of authentic standards  
272 purchased from commercial vendors.

273 *Statistical Analysis.* Data were analyzed by the GLM procedure of  
274 SAS (Cary, NC). The relationships between experimental dependent  
275 variables and S treatments were determined by regression analysis.  
276 Orthogonal polynomials were also used to study changes associated  
277 with decreasing S levels by partitioning the sums of squares into  
278 components associated with linear and quadratic terms (31).

## RESULTS AND DISCUSSION

279  
280 *Plant Growth.* Differences in shoot fresh weight (SFW;  $F = 5.64$ ,  $P < 0.001$ ) and dry weight (SDW;  $F = 4.89$ ,  $P < 0.001$ ) were found among the cultivars (data not shown). However, no differences in SFW or SDW were measured for S treatments or the interaction of S and cultivar. The nonsignificance of SFW and SDW among the kale cultivars in response to increasing S treatments follows previously reported trends in other *Brassica* crops. Matula and Zuckalová (32) reported that dry matter yield of oilseed rape (*Brassica napus* L.) was not affected by increasing MgSO<sub>4</sub> fertility in potted soil culture. Hara and Sonoda (33) also observed no differences in SFW and SDW in cabbage (*Brassica oleracea* L. Capitata Group) grown in nutrient solution culture at 10 and 100 mg of S/L. Yield considerations, therefore, should not be noteworthy when manipulating S among the range provided in this study.

295 *Mineral Elements.* Accumulation of S (%S) in leaves  
296 responded significantly to S treatment concentration ( $F = 113.25$ ,  $P < 0.001$ ), cultivar ( $F = 22.07$ ,  $P < 0.001$ ), and the interaction of S treatment and cultivar ( $F = 2.33$ ,  $P < 0.044$ ). Leaf %S increased linearly for all cultivars [%S = 0.20 + 0.40(trt) for Winterbor, %S = 0.15 + 0.27(trt) for Redbor, and %S = 0.17 + 0.30(trt) for Toscano] in response to increasing S content in nutrient solution (Table 1). S levels in the leaves ranged from 0.18% for Redbor at 4 mg of S/L to 1.79% for Winterbor in response to 64 mg of S/L. The reported leaf S sufficiency range of most plants is between 0.15 and 0.50% (2). Kastori et al. (34) reported that when the S level was increased from 0 to 96 mg of S/L in nutrient solution culture, the level of leaf tissue S of sugar beet increased 1100%. Increasing the concentration of S in the nutrient solution from 4 to 64 mg of S/L increased leaf tissue S levels of kale 716% in Winterbor, 622% in Redbor, and 638% in Toscano.

312 Concentrations of other mineral elements were slightly above  
313 sufficiency ranges previously reported in leaves of mature, field-  
314 grown kale (data not shown) (35). Only levels of Mg and Ca,  
315 however, were significantly affected by S availability in the  
316 nutrient solutions. The level of leaf Mg (%Mg) responded  
317 significantly to S treatment ( $F = 11.69$ ,  $P < 0.001$ ) and cultivar

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**Table 1.** Percent Macronutrient Content<sup>a</sup> of Leaf Tissue for Winterbor, Redbor, and Toscano Kale (*B. oleracea* L. Acephala Group) Cultivars Grown at Increasing Sulfur (S) Concentrations in Nutrient Solution Culture

mg of S/L	%S	%Mg	%Ca
Winterbor			
4	0.25 ± 0.03	0.82 ± 0.04	5.25 ± 0.29
8	0.46 ± 0.09	0.76 ± 0.05	4.86 ± 0.43
16	1.04 ± 0.06	0.76 ± 0.05	5.00 ± 0.06
32	1.37 ± 0.12	0.70 ± 0.06	4.30 ± 0.33
64	1.79 ± 0.06	0.72 ± 0.03	4.45 ± 0.38
contrasts			
linear	<i>P</i> < 0.001	<i>P</i> = 0.016	<i>P</i> = 0.001
quadratic	ns <sup>b</sup>	ns <sup>b</sup>	ns <sup>b</sup>
Redbor			
4	0.18 ± 0.02	0.85 ± 0.02	4.90 ± 0.38
8	0.29 ± 0.03	0.75 ± 0.02	4.35 ± 0.21
16	0.54 ± 0.04	0.69 ± 0.05	4.01 ± 0.22
32	1.09 ± 0.05	0.66 ± 0.06	3.60 ± 0.25
64	1.12 ± 0.06	0.69 ± 0.02	3.85 ± 0.33
contrasts			
linear	<i>P</i> < 0.001	<i>P</i> < 0.001	<i>P</i> < 0.001
quadratic	ns <sup>b</sup>	<i>P</i> = 0.001	<i>P</i> = 0.010
Toscano			
4	0.21 ± 0.03	1.03 ± 0.06	4.13 ± 0.26
8	0.36 ± 0.05	0.71 ± 0.05	3.70 ± 0.43
16	0.68 ± 0.09	0.80 ± 0.06	3.59 ± 0.12
32	1.13 ± 0.20	0.80 ± 0.08	3.36 ± 0.53
64	1.34 ± 0.19	0.74 ± 0.05	3.27 ± 0.51
contrasts			
linear	<i>P</i> < 0.001	ns <sup>b</sup>	<i>P</i> = 0.004
quadratic	ns <sup>b</sup>	ns <sup>b</sup>	ns <sup>b</sup>

<sup>a</sup> Percent composition of sampled leaf blade tissues of four replications, five plants each ± the standard error. <sup>b</sup> Nonsignificant.

(*F* = 7.23, *P* = 0.002), but not for the interaction of S treatment and cultivar. As S availability decreased, %Mg increased from 0.72 to 0.82% for Winterbor [%Mg = 0.81 - 0.02(trt)] and from 0.66 to 0.85% for Redbor [%Mg = 0.85 - 0.04(trt)] (Table 1). Although the Toscano leaf tissue Mg concentration increased from 0.74 to 1.03%, no trend was apparent. Accumulation of Ca (%Ca) in kale leaves responded significantly to S treatment (*F* = 21.26, *P* < 0.001) and cultivar (*F* = 75.22, *P* < 0.001), but not to the interaction of S and cultivar. As S availability decreased, leaf %Ca increased linearly for all the cultivars that were tested [%Ca = 5.52 - 0.24(trt) for Winterbor, %Ca = 4.99 - 0.28(trt) for Redbor, and %Ca = 4.13 - 0.20(trt) for Toscano] (Table 1). Lowering S availability, therefore, may improve the leaf content of these two important mineral elements.

The *Brassica* vegetables do not accumulate oxalate, a C<sub>2</sub> dicarboxylic acid, to detoxify excess Ca to protect against cell death. When consumed, oxalic acid in plants can bind with both Ca and Mg in the human intestinal track to form insoluble salts, lowering the rate of uptake (36). In fact, diets high in oxalate-rich vegetables, low in Ca-rich foods such as milk and cheese, and low in vitamin D may lead to Ca deficiency (37). Therefore, kale can be an excellent source of Ca and Mg in the diet. Interestingly, the level of intestinal absorption of Ca from members of the *Brassica* genus can equal or exceed the level of Ca absorption from milk (38). Because high S availability would lower the value of kale as a dietary Ca and Mg source, S levels should be considered in production strategies aimed at Ca and Mg enhancement of kale.

**Sulfur Compounds.** Most GS compounds were affected by decreasing S availability in the nutrient solutions. Glucoiberin

accumulation responded significantly to S treatment (*F* = 7.27, *P* < 0.001), cultivar (*F* = 17.75, *P* < 0.001), and the interaction of S with cultivar (*F* = 2.42, *P* = 0.03). As S availability decreased, significant linear decreases in glucoiberin content were measured [Glucoiberin = -3.1 + 6.3(trt) for Winterbor, Glucoiberin = -4.4 + 3.0(trt) for Redbor, and Glucoiberin = -1.0 + 0.8(trt) for Toscano] (Table 2). Glucoiberin was undetectable at the lowest S concentration for all cultivars.

Sinigrin, an aliphatic glucosinolate, accumulation responded significantly to S treatment (*F* = 2.59, *P* = 0.05) and kale cultivar (*F* = 27.87, *P* < 0.001). Sinigrin was undetectable at a concentration of 4 mg of S/L for Winterbor and Redbor, while the sinigrin content in Toscano was unaffected by S treatment (Table 2). Significant linear decreases in sinigrin content in leaf tissues were measured for Winterbor [Sinigrin = -1.2 + 6.7(trt)] and Redbor [Sinigrin = -1.3 + 0.9(trt)] in response to decreasing S availability. Similarly, Davik and Bakken (39) reported minimal detection of aliphatic GS in seeds of oilseed rape (*B. napus* L.) at low S fertility, but measured significant increases in concentration in response to increasing S levels in inbred and hybrid lines.

Glucobrassicin accumulation responded significantly to only S treatment and was the most abundant GS in all cultivars (*F* = 14.86, *P* < 0.001) (Table 2). Significant linear decreases in glucobrassicin content in leaf tissues were measured in response to decreased S availability [Glucobrassicin = 14.1 + 60.2(trt) for Winterbor, Glucobrassicin = -34.7 + 75.6(trt) for Redbor, and Glucobrassicin = 51.1 + 43.7(trt) for Toscano]. Neoglucobrassicin accumulation responded significantly only to kale cultivar (*F* = 24.36, *P* < 0.001) (Table 2). However, the trends in response to S availability were cultivar-dependent. Neoglucobrassicin content in leaf tissue linearly increased for Redbor [Neoglucobrassicin = 0.9 + 0.9(trt)], increased and then decreased quadratically for Winterbor [Neoglucobrassicin = -4.3 + 14.2(trt) - 2.2(trt<sup>2</sup>)], and decreased and then increased quadratically for Toscano [Neoglucobrassicin = 12.4 - 7.3(trt) + 1.4(trt<sup>2</sup>)].

4-Hydroxyglucobrassicin accumulation responded significantly to only S treatment (*F* = 3.62, *P* = 0.01) (Table 2). Redbor was the only cultivar whose level decreased in response to decreasing S availability [4-Hydroxyglucobrassicin = 1.3 + 1.6(trt)]. Davik and Bakken (39) reported that S supply and inbred or hybrid lines influenced the concentration of 4-hydroxyglucobrassicin in seeds of oilseed rape. 4-Methoxyglucobrassicin accumulation was only significant among kale cultivar (*F* = 31.09, *P* < 0.001).

Glucosinolate content and accumulation in vegetable Brassicas appears to be determined by S fertility, cultivar or accession, harvest time and date, and growing season. Total GS and leaf S levels in vegetable turnip rape (*Brassica rapa* L.) increased in response to increasing S fertility from 0.5 to 2.0 mM in pot culture (40). Kushad et al. (22) reported significant differences in individual and total GS accumulation among 65 different cultivars and accessions of *B. oleracea*. Rosa et al. (41) observed significant diurnal differences in the GS content in the leaves of *B. oleracea* Acephala and Capitata Groups, the lowest concentrations of which occurred between 10:00 am and 2:00 pm. Significant differences in both individual and total GS among nine harvest dates and two growing seasons in *B. oleracea* and *B. napus* have also been reported (42). In one growing season, Kushad et al. (22) reported levels of sinigrin were 5–190 times higher than levels of glucobrassicin in field-grown Vates and Winterborne kale. In contrast, our results demonstrate glucobrassicin at concentrations much higher than

## Kale Carotenoids

**Table 2.** Mean Glucosinolate (milligrams per 100 grams of dry mass) and Methylcysteine Sulfoxide (MCSO; milligrams per gram of dry mass) Content<sup>a</sup> of Leaf Tissue for Winterbor, Redbor, and Toscano Kale (*B. oleracea* L. Acephala Group) Cultivars Grown at Increasing Sulfur (S) Concentrations in Nutrient Solution Culture

mg of S/L	glucosinolates						MCSO
	glucoiberin	sinigrin	glucobrassicin	neoglucobrassicin	4-methoxygluco-brassicin	4-hydroxygluco-brassicin	
Winterbor							
4	nd <sup>b</sup>	2.1 ± 3.0	45.3 ± 7.8	9.2 ± 2.3	12.9 ± 2.9	3.9 ± 4.6	0.6 ± 0.7
8	5.6 ± 3.9	14.3 ± 8.6	104.4 ± 51.2	11.3 ± 4.4	9.9 ± 7.6	4.4 ± 4.4	2.0 ± 0.5
16	22.9 ± 8.5	22.5 ± 31.1	274.3 ± 92.9	21.8 ± 10.9	14.4 ± 2.9	6.1 ± 5.7	4.4 ± 1.1
32	25.6 ± 14.3	26.1 ± 15.6	302.4 ± 79.5	17.3 ± 9.9	13.4 ± 5.7	9.9 ± 4.1	4.9 ± 0.9
64	23.1 ± 4.9	29.8 ± 9.5	247.5 ± 78.8	11.6 ± 4.9	11.8 ± 4.6	7.3 ± 2.8	4.6 ± 1.0
contrasts							
linear	<i>P</i> < 0.001	<i>P</i> = 0.013	<i>P</i> < 0.001	ns <sup>c</sup>	ns <sup>c</sup>	<i>P</i> = 0.087	<i>P</i> < 0.001
quadratic	<i>P</i> = 0.021	ns <sup>c</sup>	<i>P</i> = 0.016	<i>P</i> = 0.039	ns <sup>c</sup>	ns <sup>c</sup>	<i>P</i> = 0.006
Redbor							
4	nd <sup>b</sup>	nd <sup>b</sup>	39.6 ± 22.1	1.2 ± 1.4	16.1 ± 10.9	2.4 ± 3.7	nd <sup>b</sup>
8	nd <sup>b</sup>	0.2 ± 0.6	94.3 ± 63.8	3.0 ± 0.9	35.2 ± 15.8	4.2 ± 3.4	1.5 ± 0.4
16	2.7 ± 3.5	0.9 ± 1.1	232.3 ± 90.4	3.9 ± 1.9	36.3 ± 6.3	7.5 ± 2.8	2.0 ± 0.2
32	10.9 ± 9.5	3.1 ± 2.9	258.9 ± 89.9	5.1 ± 3.2	37.3 ± 5.4	8.0 ± 4.8	2.0 ± 0.1
64	9.7 ± 10.3	3.3 ± 1.7	335.2 ± 268.4	4.6 ± 3.4	36.8 ± 10.8	7.1 ± 2.4	2.8 ± 0.7
contrasts							
linear	<i>P</i> = 0.007	<i>P</i> = 0.001	<i>P</i> = 0.001	<i>P</i> = 0.022	<i>P</i> = 0.024	<i>P</i> = 0.009	<i>P</i> < 0.001
quadratic	ns <sup>c</sup>	ns <sup>c</sup>	ns <sup>c</sup>	ns <sup>c</sup>	<i>P</i> = 0.056	ns <sup>c</sup>	<i>P</i> = 0.032
Toscano							
4	nd <sup>b</sup>	nd <sup>b</sup>	56.54 ± 24.7	6.5 ± 1.7	24.6 ± 7.5	1.7 ± 2.2	2.5 ± 0.3
8	0.5 ± 0.8	nd <sup>b</sup>	127.9 ± 22.1	3.5 ± 2.5	31.2 ± 14.3	6.6 ± 6.3	3.3 ± 0.1
16	1.3 ± 2.5	nd <sup>b</sup>	256.8 ± 104.5	3.1 ± 2.7	28.2 ± 8.2	5.0 ± 0.9	5.3 ± 0.2
32	2.3 ± 3.1	nd <sup>b</sup>	261.5 ± 84.7	5.3 ± 4.6	29.8 ± 13.9	4.8 ± 3.2	4.6 ± 0.3
64	3.2 ± 4.0	nd <sup>b</sup>	208.1 ± 100.4	10.6 ± 7.7	25.6 ± 18.4	7.1 ± 2.4	5.0 ± 0.8
contrasts							
linear	<i>P</i> = 0.042	nd <sup>b</sup>	<i>P</i> = 0.005	ns <sup>c</sup>	ns <sup>c</sup>	ns <sup>c</sup>	<i>P</i> < 0.001
quadratic	ns <sup>c</sup>	nd <sup>b</sup>	<i>P</i> = 0.017	<i>P</i> = 0.023	ns <sup>c</sup>	ns <sup>c</sup>	<i>P</i> = 0.006

<sup>a</sup> Composition of sampled leaf blade tissues of four replications, five plants each ± the standard error. <sup>b</sup> Nondetectable. <sup>c</sup> Nonsignificant.

413 that of sinigrin among all the S treatment levels among the kale  
414 cultivars (Table 3). Ciska et al. (43) reported levels of  
415 glucobrassicin 4 and 20 times higher than that of sinigrin in  
416 field-grown Srednio Wysoki Zielony kale in growing seasons  
417 1 and 2, respectively. In that same study, sinigrin levels  
418 increased from 2.21 to 22.72 mg/100 g of dry mass and  
419 glucobrassicin levels increased from 43.44 to 92.13 mg/100 g  
420 of dry mass from season 1 to season 2 in response to the  
421 decreased rainfall and increased growing temperatures experi-  
422 enced in season 2 (43). Therefore, many environmental and  
423 cultural factors need to be considered in plant improvement  
424 strategies focusing on glucosinolate accumulation.

425 Methylcysteine sulfoxide was affected by decreasing S  
426 concentrations in nutrient solution ( $F = 75.17$ ,  $P < 0.001$ ), by  
427 cultivar ( $F = 111.69$ ,  $P < 0.001$ ), and by the interaction of S  
428 with cultivar ( $F = 5.06$ ,  $P = 0.002$ ). MCSO content decreased  
429 linearly in all the cultivars in response to decreasing S  
430 availability [MCSO = 0.01 + 1.09(trt) for Winterbor, MCSO  
431 = 0.16 + 0.61(trt) for Redbor, and MCSO = 2.24 + 0.64(trt)  
432 for Toscano] (Table 2). Decreases in levels of GS and MCSO  
433 compounds paralleled decreases in leaf tissue %S.

434 Cellular disruption in *Brassica* results in the release of a  
435 cysteine lyase enzyme (EC 4.4.1.6) and a subsequent  $\alpha,\beta$ -  
436 elimination of the S-oxide from MCSO, giving rise to volatile  
437 and odorous thiosulfonates and low-molecular weight disulfide  
438 compounds (3). The thiosulfonates give rise to creamy, sulfury,  
439 and cabbage-like flavors with a 0.1 ppm taste panel threshold  
440 level (44). Increases in MCSO levels in response to increasing  
441 S treatment levels would be expected to enhance the flavor  
442 attributes associated with consuming raw kale. Increasing S

fertility has been reported to increase the total amount of  
S-substituted cysteine sulfoxides in onion bulbs (*Allium cepa*  
L.) and their flavor potential (27).

**Carotenoid Compounds.** Lutein,  $\beta$ -carotene, chlorophyll *a*,  
and chlorophyll *b* pigments differed among kale cultivars only  
( $F = 39.6$  and  $P < 0.0001$ ,  $F = 21.4$  and  $P < 0.0001$ ,  $F =$   
 $32.9$  and  $P < 0.0001$ , and  $F = 37.7$  and  $P < 0.0001$ ,  
respectively). However, no responses to decreasing S concentra-  
tions in nutrient solutions were noted for any carotenoid (Table  
3). Values found for lutein,  $\beta$ -carotene, chlorophyll *a*, and  
chlorophyll *b* content were within previously reported ranges  
for field-grown kale (23). In previous research, sugar beet (*Beta*  
*vulgaris* L.) leaf chlorophyll content (milligrams per gram of  
fresh mass) showed no response to increasing S levels when  
supplied at concentrations of 24–48 mg of S/L in perlite media  
(45). In a similar study, sugar beet leaf chlorophyll and  
carotenoid accumulations did not respond to treatments with  
32 and 96 mg of S/L (34).

Lutein is only one of two dietary carotenoids selectively  
deposited in the human retina and lens (46). In the retina, lutein  
is responsible for the yellow pigmentation termed macular  
pigment (MP; 47). Macular pigment is postulated to participate  
in photoprotection, and diminished MP levels may be related  
to retinal damage (48, 49). Increases in MP levels can be  
achieved through diet (50) and supplementation (51). However,  
studies have indicated that consumption of a variety of  
vegetables providing a mixture of carotenoids was more strongly  
associated with reduced eye disease and cancer risk than  
individual carotenoid supplements (16, 52). Consumption of  
vegetable carotenoids may prove to be effective at delaying the

**Table 3.** Mean Pigment Content<sup>a</sup> (milligrams per 100 GFW) of Leaf Tissue for Winterbor, Redbor, and Toscano Kale (*B. oleracea* L. Acephala Group) Cultivars Grown at Increasing Sulfur (S) Concentrations in Nutrient Solution Culture

mg of S/L	pigment (mg/100 GFW)			
	lutein	$\beta$ -carotene	chlorophyll <i>a</i>	chlorophyll <i>b</i>
Winterbor				
4	10.1 ± 0.6	8.8 ± 1.5	214.1 ± 30.9	65.3 ± 9.3
8	10.1 ± 1.3	8.7 ± 0.9	233.1 ± 25.8	65.0 ± 8.3
16	9.6 ± 1.7	8.6 ± 1.2	220.8 ± 36.1	63.5 ± 6.9
32	10.1 ± 1.4	8.9 ± 0.6	221.5 ± 27.6	65.1 ± 4.9
64	10.1 ± 0.7	8.5 ± 0.6	223.1 ± 18.7	63.8 ± 6.6
contrasts				
linear	ns <sup>b</sup>	ns <sup>b</sup>	ns <sup>b</sup>	ns <sup>b</sup>
quadratic	ns <sup>b</sup>	ns <sup>b</sup>	ns <sup>b</sup>	ns <sup>b</sup>
Redbor				
4	10.8 ± 1.7	9.8 ± 1.4	206.5 ± 44.2	72.3 ± 12.8
8	11.4 ± 1.8	9.7 ± 1.7	233.3 ± 41.5	75.3 ± 12.9
16	11.5 ± 2.2	10.4 ± 1.7	252.0 ± 48.9	80.6 ± 14.9
32	10.5 ± 1.7	8.7 ± 0.8	216.9 ± 26.6	68.9 ± 7.7
64	11.7 ± 2.4	9.6 ± 1.8	244.5 ± 34.1	78.4 ± 14.2
contrasts				
linear	ns <sup>b</sup>	ns <sup>b</sup>	ns <sup>b</sup>	ns <sup>b</sup>
quadratic	ns <sup>b</sup>	ns <sup>b</sup>	ns <sup>b</sup>	ns <sup>b</sup>
Toscano				
4	16.1 ± 2.9	12.8 ± 3.3	328.4 ± 74.1	105.7 ± 27.5
8	16.2 ± 3.3	12.0 ± 1.5	311.1 ± 49.7	98.8 ± 13.2
16	16.9 ± 2.6	13.6 ± 3.4	376.8 ± 95.0	111.4 ± 20.3
32	14.0 ± 3.2	11.1 ± 3.4	292.6 ± 76.7	91.5 ± 20.1
64	14.9 ± 1.6	11.8 ± 2.3	317.3 ± 63.1	101.3 ± 23.8
contrasts				
linear	ns <sup>b</sup>	ns <sup>b</sup>	ns <sup>b</sup>	ns <sup>b</sup>
quadratic	ns <sup>b</sup>	ns <sup>b</sup>	ns <sup>b</sup>	ns <sup>b</sup>

<sup>a</sup> Composition of sampled leaf blade tissues of four replications, five plants each ± the standard error. <sup>b</sup> Nonsignificant.

473 normal effects of aging on retinal function and, in some cases,  
 474 the progression of retinal changes resulting in age-related  
 475 macular degeneration.  $\beta$ -Carotene is biologically cleaved to  
 476 produce retinol (vitamin A), required for vision, epithelia  
 477 maintenance, secretion of mucus, and reproduction (53). Lower  
 478 S fertility in kale production would provide more palatable raw  
 479 produce, while still providing beneficial dietary lutein and  
 480  $\beta$ -carotene.

481 Consumers often cite the bitter, astringent flavor of raw  
 482 *Brassica* vegetables as unpleasant or objectionable (8, 54). To  
 483 foster consumer acceptance, it is important to consider the  
 484 sensory response to foods when developing strategies aimed at  
 485 improving dietary quality (54). The lower S treatment levels in  
 486 this study reduced GC and MCSO content in kale which should  
 487 weaken the bitter and unpleasant flavors associated with eating  
 488 raw *Brassica*. Lowering the levels of GS compounds in kale,  
 489 however, would be expected to decrease the health benefits  
 490 associated with the isothiocyanates produced from GS decom-  
 491 position. Lowering S fertility in this study did not significantly  
 492 reduce lutein and  $\beta$ -carotene levels, thereby preserving the health  
 493 benefits of carotenoid consumption. These results suggest  
 494 lowering S fertility in kale production will provide more  
 495 palatable raw produce, with enhanced Ca and Mg content, but  
 496 will not affect the levels of beneficial dietary lutein and  
 497  $\beta$ -carotene. Understanding the combined impact of fertility on  
 498 flavor compounds, carotenoid pigments, and elemental content  
 499 may help improve consumer acceptance of phytonutritionally  
 500 enhanced vegetable crops.

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