

Increase of Bioactive Sulforaphane and Its Related Compounds with Sulfur Compounds in Broccoli (*Brassica Oleracea Var. Italica*) Sprouts During Cultivation

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Abstract – The effects of sulfur containing compounds on the level of biologically active sulforaphane and related compounds in broccoli sprouts were investigated. Sulforaphane and 7 related compounds were identified in the chloroform extract of broccoli sprouts grown in cultivation waters containing different concentrations of Na₂SO₃, Na₂SO₄, or NaCl. Of the 5 nitriles identified, the concentrations of 5-methylsulfinylpentynitrile were the greatest, ranging from 2,828.2 µg g⁻¹ (grown in a 1.0 mM Na₂SO₄ cultivation water) to 825.9 µg g⁻¹ (2.0 mM NaCl). Of the 3 isothiocyanates (erucin, iberin, and sulforaphane) identified, the concentrations of sulforaphane were the greatest, ranging from 1,600.5 µg g⁻¹ (1 mM Na₂SO₄) to 453.7 µg g⁻¹ (2.0 mM NaCl), followed by iberin, which ranged from 1,001.4 ± 103.4 µg g⁻¹ (1.0 mM Na₂SO₄) to 128.7 ± 19.0 µg g⁻¹ (2.0 mM NaCl), and erucin, which ranged from 29.8 ± 3.7 µg g⁻¹ (1.0 mM Na₂SO₄) to 10.9 ± 7.7 µg g⁻¹ (2.0 mM NaCl). When fresh broccoli sprout samples were treated with myrosinase, the sulforaphane concentrations increased from 884.3 µg g⁻¹ to 5,403.6 µg/g in the sprouts grown in a 1.0 mM Na₂SO₃ cultivation water (3.78 fold). The greatest rate of increase was observed in the case of control samples. It increased from 482.5 µg g⁻¹ to 1,905.7 µg g⁻¹ (3.95 fold). The study demonstrated that the addition of sulfur compounds to cultivation water significantly increased the amounts of sulforaphane and related compounds in broccoli sprouts. In particular, myrosinase treatment increased the level of sulforaphane in broccoli sprouts; appreciable level changes were not observed for the other compounds.

Keywords - Broccoli Sprouts, Isothiocyanates, Myrosinase, Nitriles, Sulforaphane, Sulfur Supplement.

I. INTRODUCTION

Recently, natural plants have received considerable attention as sources of alternative medicines. It has been epidemiologically and experimentally demonstrated that some naturally occurring chemicals in fruits and vegetables can reduce the risk of chronic diseases such as cancers, arthritis, inflammation, diabetes, and atherosclerosis [1, 2]. Among the chemicals in the plants studied from this perspective, glucosinolates, found in cruciferous vegetables such as cabbage, broccoli, cauliflower, and Brussels sprouts, may be the most intensively studied for their biological activities [3]. In particular, there are many reports on the disease preventive activities of glucosinolates found in broccoli (*Brassica oleracea var. italica*) and broccoli sprouts [4]. The main

glucosinolate in broccoli sprouts is glucoraphanin (4-methylsulphinylbutylglucosinolate), the level of which in sprouts is 15 times higher than in mature plants. Accordingly, the content of sulforaphane, one of the secondary products of glucoraphanin, in sprouts is 10 times higher than in mature plants [5].

Glucosinolates themselves are biologically inactive but they produce active compounds by action of the enzyme myrosinase (thioglucoside glucohydrolase), which is found at relatively high levels in broccoli [6]. For example, glucosinolates, including glucoraphanin, do not possess antiproliferative activity against cancer cells but their hydrolysis products (isothiocyanates and nitriles) display potent activity [7].

When cells of broccoli or its sprouts are damaged, glucoraphanin is hydrolyzed into glucose and secondary products, including isothiocyanates and nitriles, by myrosinase [8, 9]. Among the secondary products, isothiocyanates, in particular sulforaphane, have been shown to have cancer preventive activity [10]. It has been reported that sulforaphane induces phase II enzymes which possess inhibitory effects against carcinogens and other toxic free radicals [11]. A sulforaphane analog, 6-methylsulfinylhexyl isothiocyanate present in wasabi (*Wasabia japonica Matsum.*), was found to exhibit anti-inflammatory activity [12]. Nitriles yielded from glucoraphanin reportedly exhibit anti-bacterial activity [13] and anti-inflammatory activity [14].

In the present study, the effect of sulfur sources (Na₂SO₃ and Na₂SO₄) on the concentration of sulforaphane and related compounds in broccoli sprouts was investigated in order to develop methods for preparing a natural plant medicine with high levels of sulforaphane. Broccoli sprouts were chosen because they contain higher levels of glucoraphanin [15] and sulforaphane than the mature plants [5].

II. MATERIALS AND METHODS

A. Chemicals and materials

n-Hexane, chloroform, and water (HPLC-grade) were purchased from Fisher Scientific Co. (Rochester, NY, USA). 4-Methylsulfinylbutylisothiocyanate (sulforaphane) and related compounds (3-methylthioalkylisothiocyanates, 3-methylthioalkylnitriles, 3-methylsulfinylalkylisothio

cyanates, and 2-methylsulfinyl alkylnitriles) were synthesized by a previously reported method [13]. Myrosinase (thioglucosidase) from *Sinapis alba* (white mustard) seed (≥ 100 units g^{-1} solid) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Broccoli (*Brassica oleracea* var. *300italic*) seeds were bought from Todd's seeds (Novi, MI, USA).

B. Cultivation of broccoli sprouts

Sprouts were grown in a room maintained in darkness at 22 °C. Seeds of broccoli (*Brassica oleracea* var. *300italic*) (50 g) were soaked in distilled water for 2 h and then placed in an 18 cm-diameter circular tray (Handy Pantry, Springville, UT, USA). The tray was placed on another tray filled with distilled water to maintain saturated humidity. The cultivation water solution was changed from distilled water to solutions with different contents (2.0 mM NaCl, 0.1 mM Na_2SO_3 , 1.0 mM Na_2SO_3 , 0.1 mM Na_2SO_4 , or 1 mM Na_2SO_4) after 48 h. One tray with distilled water alone was used as a control. The height of water in the trays was 1 mm, a level at which only the roots of the sprouts were in the water. Each experimental solution was replaced with a fresh solution twice a day. The sprouts were harvested after 5 days.

The weight of the freshly collected broccoli sprouts was 200 ± 5 g (water only, control), 189 ± 36 g (2.0 mM NaCl solution), 221 ± 35 g (0.1 mM Na_2SO_3 solution), 196 ± 6 g (1.0 mM Na_2SO_3 solution), 201 ± 6 g (0.1 mM Na_2SO_4 solution), and 205 ± 13 g (1.0 mM Na_2SO_4 solution).

C. Sample preparations for analysis of sulforaphane and related compounds

Sprout extracts were prepared according to the previously reported method cited above [13]. Fresh broccoli sprouts (50 g) grown under the different cultivation conditions described above were soaked in 150 mL of boiling water for 5 min to inactivate endogenous myrosinase and then allowed to stand until cooled to 37 °C. After heat treatment, the sprouts were homogenized in a blender (Waring, New Hartford, CT, USA) for 10 min. Myrosinase (20 units) was added to the 150 mL homogenized sprout solution and then incubated at 35 °C for 3.5 h according to a previously reported method [7].

The 150 mL each of homogenized myrosinase non-treated-solutions and treated solutions was centrifuged to remove solid materials for 1 min at 5000 g. Each resulting supernatant (adjusted to 500 mL with water) was extracted with 500 mL *n*-hexane to remove chlorophyll and then with 500 mL of chloroform using a separatory funnel. Each chloroform extract was then condensed to approximately 1 mL in volume using a rotary evaporator. The solvent was further removed under a purified nitrogen gas stream to yield brown viscous material. The weights of the viscous material from myrosinase non-treated samples was 91.9 mg from the control, 60.5 mg from NaCl, 71.4 mg from 0.1 mM Na_2SO_3 , 80.4 mg from 1 mM Na_2SO_3 , 86.8 mg from 0.1 mM Na_2SO_4 , and 84.8 mg from 1 mM Na_2SO_4 . The weights of the viscous material from the myrosinase treated samples was 10.0 mg from the control, 90.0 mg from NaCl, 80.0 mg from 0.1 mM Na_2SO_3 , 87.9 mg from 1 mM Na_2SO_3 , 57.9 mg from 0.1 mM Na_2SO_4 , and 75.6 mg from 1 mM Na_2SO_4 . Each sample of viscous material

was dissolved into ethyl acetate to prepare a 100 mg mL^{-1} sample solution for the quantitative analysis of sulforaphane and related compounds.

D. Identification and quantification of sulforaphane and related compounds

The ethyl acetate solutions prepared above were analyzed for sulforaphane and related compounds using an Agilent 6890 GC equipped with a 30 m x 0.25 mm i.d. ($d_f = 0.5 \mu\text{m}$) DB-5 bonded-phase fused silica capillary column (Agilent, Folsom, CA, USA) and a nitrogen phosphorus detector (NPD). The helium carrier gas flow rate was 1.5 mL min^{-1} at a split ratio of 20:1. The injector and detector temperatures were 250 °C and 280 °C, respectively. The oven temperature was programmed from 50 °C to 280 °C at 2 °C min^{-1} . An Agilent model 6890 GC interfaced to an Agilent 5971A mass selective detector (GC/MS) was used for mass spectral identification of the GC components at MS ionization voltage of 70 eV. GC column conditions were exactly the same as the ones used for GC/NPD. A standard curve for quantitative analysis of Compounds **I–VIII** was prepared using three concentrations (100 $\mu\text{g mL}^{-1}$, 1,000 $\mu\text{g mL}^{-1}$, and 10,000 $\mu\text{g mL}^{-1}$) of each authentic chemical. R^2 value of standard curves ranged from 1.00000 (Compounds **VI** and **VIII**) to 0.99848 (Compound **II**).

The identification of sulforaphane and related compounds was performed by comparing the Kovats gas chromatographic retention index and the mass spectral fragmentation pattern of each GC component to those of authentic compounds. The identification of the chemicals was also confirmed with the NIST AMDIS version 2.1 software.

E. Statistical analysis

The results were expressed as the mean \pm SD ($n = 3$). Data were analyzed by one-way ANOVA, followed by *Dunnnett's* test for comparison with the control group. Differences were considered significant at a *P* value of < 0.05 .

III. RESULTS AND DISCUSSION

A typical gas chromatogram of a chloroform extract from a control sample is shown in Fig. I. Fig. II shows the structures and gas chromatographic Kovats indexes of the sulforaphane and related compounds identified in the chloroform extract from broccoli sprouts. The analytical results of sulforaphane and related compounds are shown in $\mu\text{g/g}$ of fresh broccoli sprouts in the figures.

Fig.III shows the analytical results of methylthio alkylnitriles (Compounds **I**, **II**, and **III**) in the chloroform extracts from various sprout samples grown in different cultivation waters. The concentration of Compound **III** was the greatest, ranging from $547.3 \pm 97.7 \mu\text{g g}^{-1}$ (grown in a 1.0 mM Na_2SO_4 cultivation water) to $142.8 \pm 71.5 \mu\text{g g}^{-1}$ (0.1 mM Na_2SO_3); followed by Compound **II** ranging from $174.2 \pm 11.3 \mu\text{g g}^{-1}$ (1.0 mM Na_2SO_3) to $101.7 \pm 2.3 \mu\text{g g}^{-1}$ (2.0 mM NaCl); and Compound **I** ranging from $52.0 \pm 32.1 \mu\text{g g}^{-1}$ (1.0 mM Na_2SO_4) to $19.6 \pm 0.5 \mu\text{g g}^{-1}$ (2.0 mM NaCl). The concentration of Compound **II** increased considerably upon addition of 1.0 mM Na_2SO_3 ($P =$

0.0013) or 1.0 mM Na₂SO₄ ($P = 0.0072$) to the cultivation water. Also, the concentration of Compound **III** increased approximately 3-fold upon addition of 1.0 mM Na₂SO₃ or 1.0 mM Na₂SO₄.

Fig.IV shows the analytical results of the methylsulfinylalkylnitriles (Compounds **IV** and **VI**) in the chloroform extracts. The concentration of Compound **IV** showed no appreciable changes after the addition of Na₂SO₃ or Na₂SO₄ at the levels tested. The concentration of Compound **VI** ranged from $2,828.2 \pm 190.0 \mu\text{g g}^{-1}$ (1.0 mM Na₂SO₄) to $825.9 \pm 96.0 \mu\text{g g}^{-1}$ (2.0 mM NaCl). The concentration of **VI** increased approximately 2- and 3-fold after the addition of 1.0 mM Na₂SO₃ and 1.0 mM Na₂SO₄, respectively, whereas the addition of these at the lower level of 0.1 mM did not show any changes.

NaCl was used to examine a role of Na⁺ in the formation of sulforaphane and related compounds. The results indicated that generally, the addition of relatively high concentrations of NaCl (2.0 mM) to cultivation water slightly reduced the level of compounds in broccoli sprouts but the values were not significant. Therefore, sulfite and sulfate work as a sulfur source without being significantly influenced by Na⁺.

Fig.V shows the analytical results of isothiocyanates erucin (Compound **V**) and iberin (Compound **VII**) in the chloroform extracts. The concentration of Compound **V** ranged from $29.8 \pm 3.7 \mu\text{g g}^{-1}$ to $10.9 \pm 7.7 \mu\text{g g}^{-1}$. An appreciable increase was observed in the concentration of Compound **V** when 1.0 mM Na₂SO₃ ($P = 0.0309$), 0.1 mM Na₂SO₄ ($P = 0.0245$), or 1.0 mM Na₂SO₄ ($P = 0.0132$) was added. Also, the concentration of Compound **VII**, ranging from $1001.4 \pm 103.4 \mu\text{g g}^{-1}$ to $128.7 \pm 19.0 \mu\text{g g}^{-1}$, increased significantly after the addition of 1.0 mM Na₂SO₃ ($P < 0.0001$), 0.1 mM Na₂SO₄ ($P = 0.009$), or 1.0 mM Na₂SO₄ ($P < 0.0001$).

Fig.VI shows the analytical results of sulforaphane (Compound **VIII**) in the chloroform extracts from sprout samples before and after myrosinase treatment. The analytical results of the compounds beside sulforaphane did not show appreciable differences in the samples before and after myrosinase treatment. However, it should be noted that the increase of sulforaphane content is important because it has been reported to possess potent biological activities, such as anti-carcinogenic effects [16]. In the case of samples before myrosinase treatment, the concentration of sulforaphane ranged from $1,600.5 \pm 195.1 \mu\text{g g}$ (1.0 mM Na₂SO₄) to $453.7 \pm 50.3 \mu\text{g g}^{-1}$ (2.0 mM NaCl). An obvious increase in sulforaphane concentration was observed when 1.0 mM Na₂SO₃ ($P < 0.0001$) or 1.0 mM Na₂SO₄ ($P = 0.0403$) was added. In the case of samples after myrosinase treatment, the concentration of sulforaphane ranged from $5,403.6 \pm 951.9 \mu\text{g g}^{-1}$ (1.0 mM Na₂SO₃) to $1,905.7 \pm 40.5 \mu\text{g g}^{-1}$ (control). In particular, a significant increase was observed when 1.0 mM Na₂SO₃ ($P = 0.0009$) or 1.0 mM Na₂SO₄ ($P = 0.0025$) was added. It is obvious that myrosinase treatment increased sulforaphane formation significantly. The increasing rate of sulforaphane formation by myrosinase treatment ranged from 4.3-fold (2.0 mM NaCl) to 1.8-fold (0.1 mM Na₂SO₄).

Fig.VII shows the proposed formation pathways of the isothiocyanates and nitriles found in broccoli sprouts from corresponding glucosinolate in the present study. In the case of sulforaphane, myrosinase breaks the β -thioglucoside bond of glucoraphanin molecule to produce glucose and an unstable intermediate. Subsequently sulforaphane is formed from this intermediate *via* a Lossen type rearrangement [17]. Nitriles, such as Compounds **I**, **II**, **III**, and **VI**, are also known to form from glucosinolates. However, this enzymatic pathway has not been well established yet, although numerous possible mechanisms have been reported. One article reported that their formation was catalyzed by Fe²⁺ involving an OH group at C-2 in the glucose side chain of a glucosinolate [18].

It has been reported that the biosynthesis of glucoraphanin and its hydrolysis products, including sulforaphane, is influenced by various factors (temperature, pH, nitrogen/sulfur availability, the presence of metals, and water quality) during the germination of broccoli sprouts [16, 19, 20]. One previous study reported that the addition of sulfur increased glucosinolates in cruciferous vegetables [21], suggesting that sulfur plays a role in the concentration of glucoraphanin and its hydrolysis products in broccoli sprouts. Another study reported that supplying K₂SO₄ increased the level of glucosinolates in broccoli sprouts [22]. Sulfur fertilization has also been shown to increase the levels of glucosinolates in broccoli [22, 23]. However, factors influencing the formation of sulforaphane and related compounds are not well understood yet. The addition of sulfur sources (Na₂SO₃ and Na₂SO₄) in the cultivation water increased sulforaphane and related compounds considerably in the present study, suggesting that sulfur increases endogenous glucoraphanin synthesis during the germination of broccoli sprouts and subsequently increases secondary products including sulforaphane.

The present study demonstrates that germination conditions are important for the secondary metabolite composition of broccoli sprouts. The simple addition of sulfur containing compounds to the cultivation water for sprouts germination provided broccoli sprouts with high levels of health beneficial sulforaphane and related compounds. Moreover, the preparation of sprouts is simple and requires only a short time (2 weeks). Broccoli sprouts may be an excellent source of bioactive chemicals which prevent various diseases and consequently are beneficial to human health.

IV. CONCLUSION

The present study demonstrates that germination conditions are important for the secondary metabolite composition of broccoli sprouts. The simple addition of sulfur containing compounds to the cultivation water for sprouts germination provided broccoli sprouts with high levels of health beneficial sulforaphane and related compounds. Moreover, the preparation of sprouts is simple and requires only a short time (2 weeks). Broccoli sprouts may be an excellent source of bioactive chemicals which

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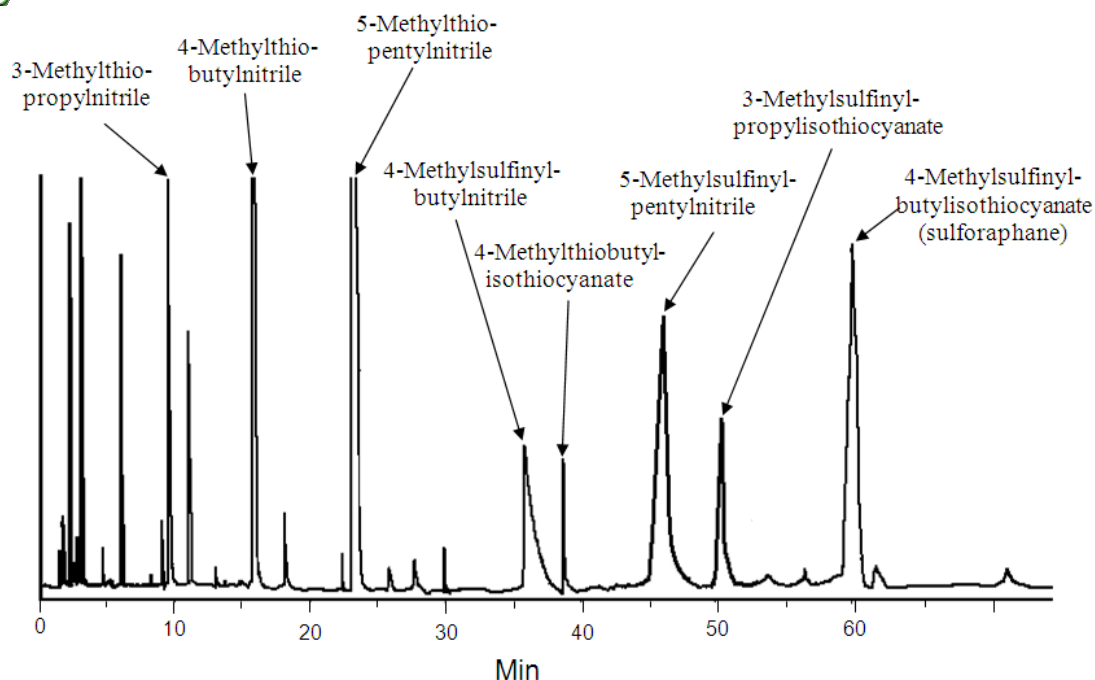


Fig.I. A typical gas chromatogram of a chloroform extract from the control sample.

No.	Compound	Structure	Kovats Index (DB-5)
I	3-Methylthiopropylnitrile	<chem>CSCCC#N</chem>	929
II	4-Methylthiobutyl nitrile	<chem>CSCCCC#N</chem>	1028
III	5-Methylthiopentyl nitrile	<chem>CSCCCCC#N</chem>	1156
IV	4-Methylsulfinylbutyl nitrile	<chem>CS(=O)CCCC#N</chem>	1379
V	4-Methylthiobutylisothiocyanate (Erucin)	<chem>CSCCCCN=C=S</chem>	1440
VI	5-Methylsulfinylpentyl nitrile	<chem>CS(=O)CCCCC#N</chem>	1518
VII	3-Methylsulfinylpropylisothiocyanate (Iberin)	<chem>CS(=O)CCCN=C=S</chem>	1612
VIII	4-Methylsulfinylbutylisothiocyanate (Sulforaphane)	<chem>CS(=O)CCCCN=C=S</chem>	1753

Fig.II. Name and structure of compounds identified in the present study along with their Kovats Index

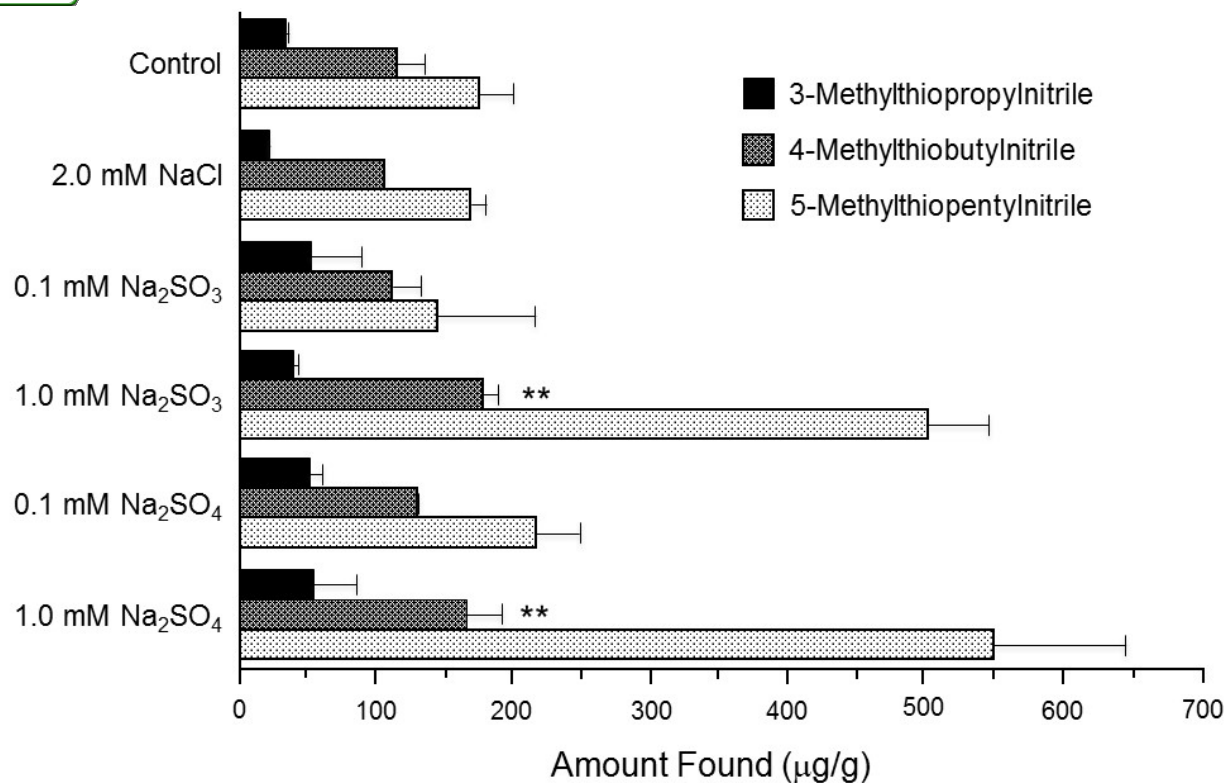


Fig.III: Analytical results of methylthioalkylnitriles (Compounds I, II, and III) in the chloroform extracts from various sprout samples grown in different cultivation waters. Values are means \pm SD (n = 3). **: p < 0.01.

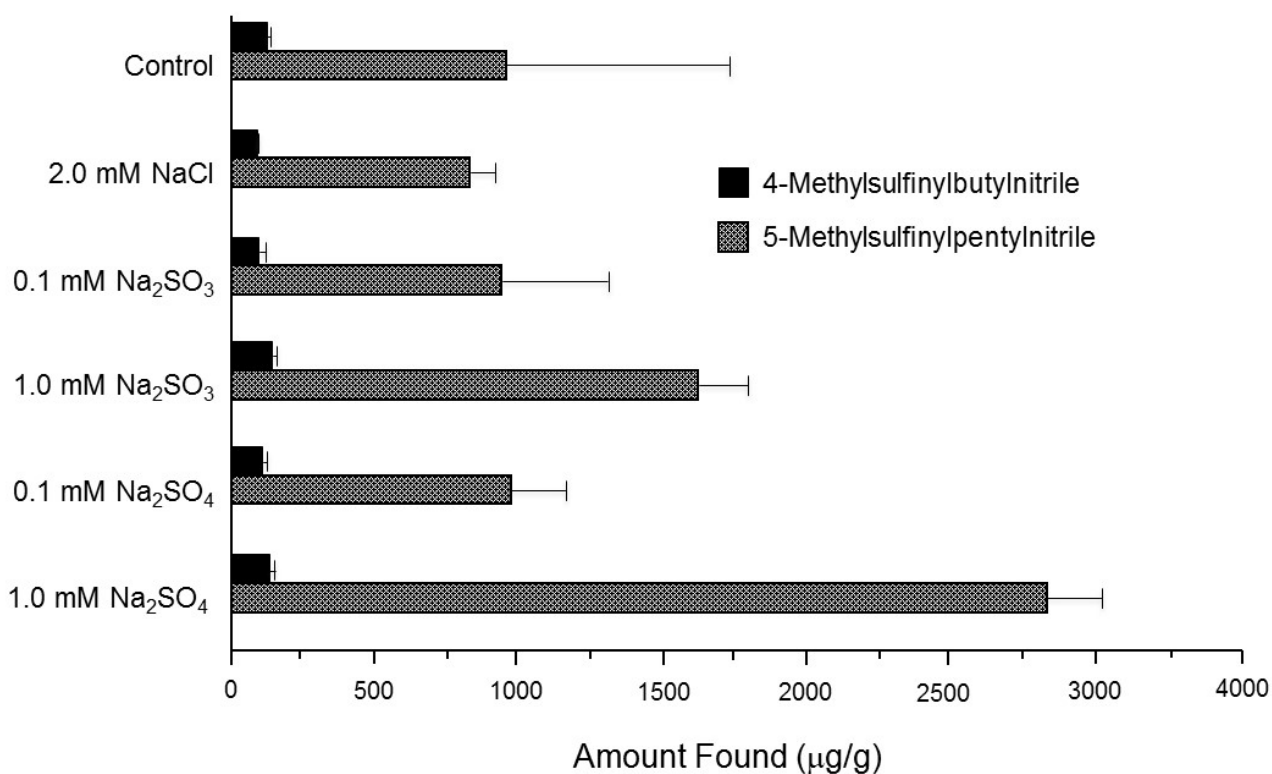


Fig.IV. Analytical results of methylsulfinylalkylnitriles (Compounds IV and VI) in the chloroform extracts from various sprout samples grown in different cultivation waters. Values are means \pm SD (n = 3)

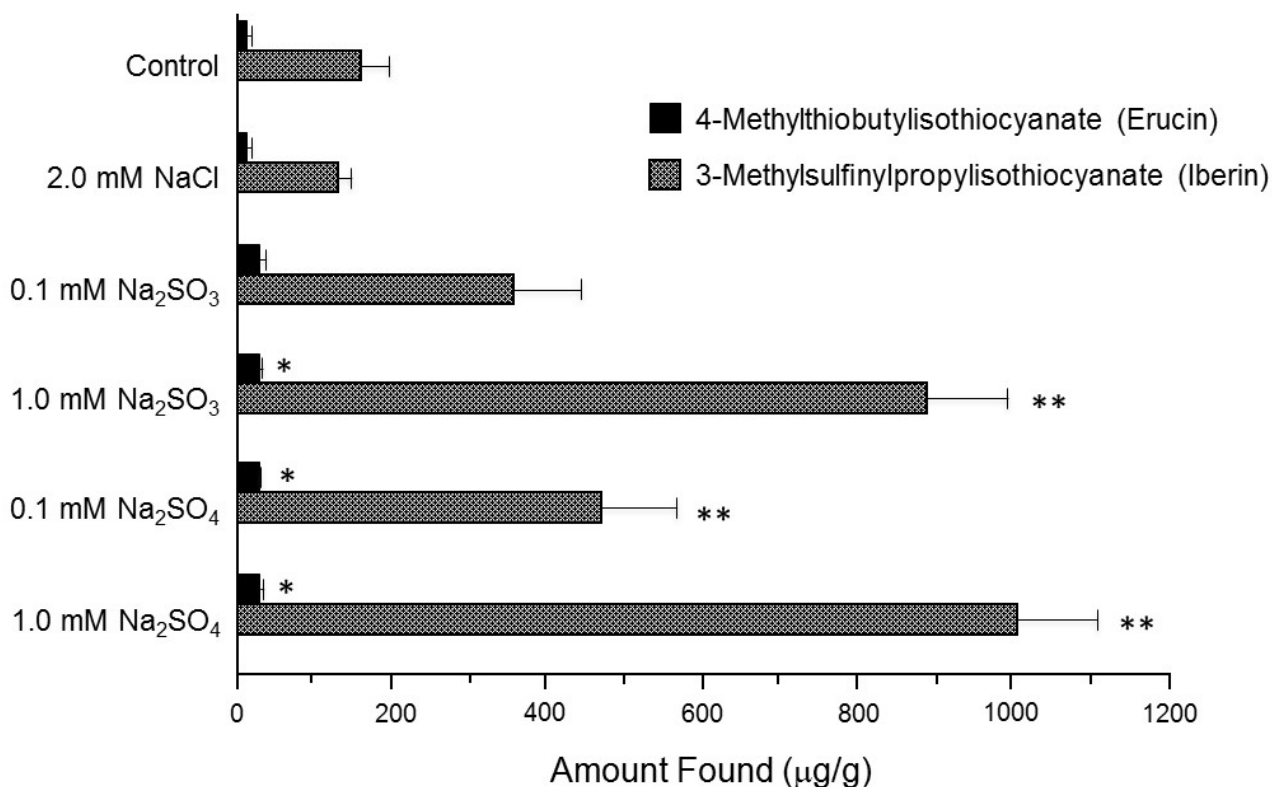


Fig.V. Analytical results of erucin and iberin (Compounds V and VII) in the chloroform extracts from various sprout samples grown in different cultivation waters. Values are means \pm SD (n = 3). *: p < 0.05, **: p < 0.01.

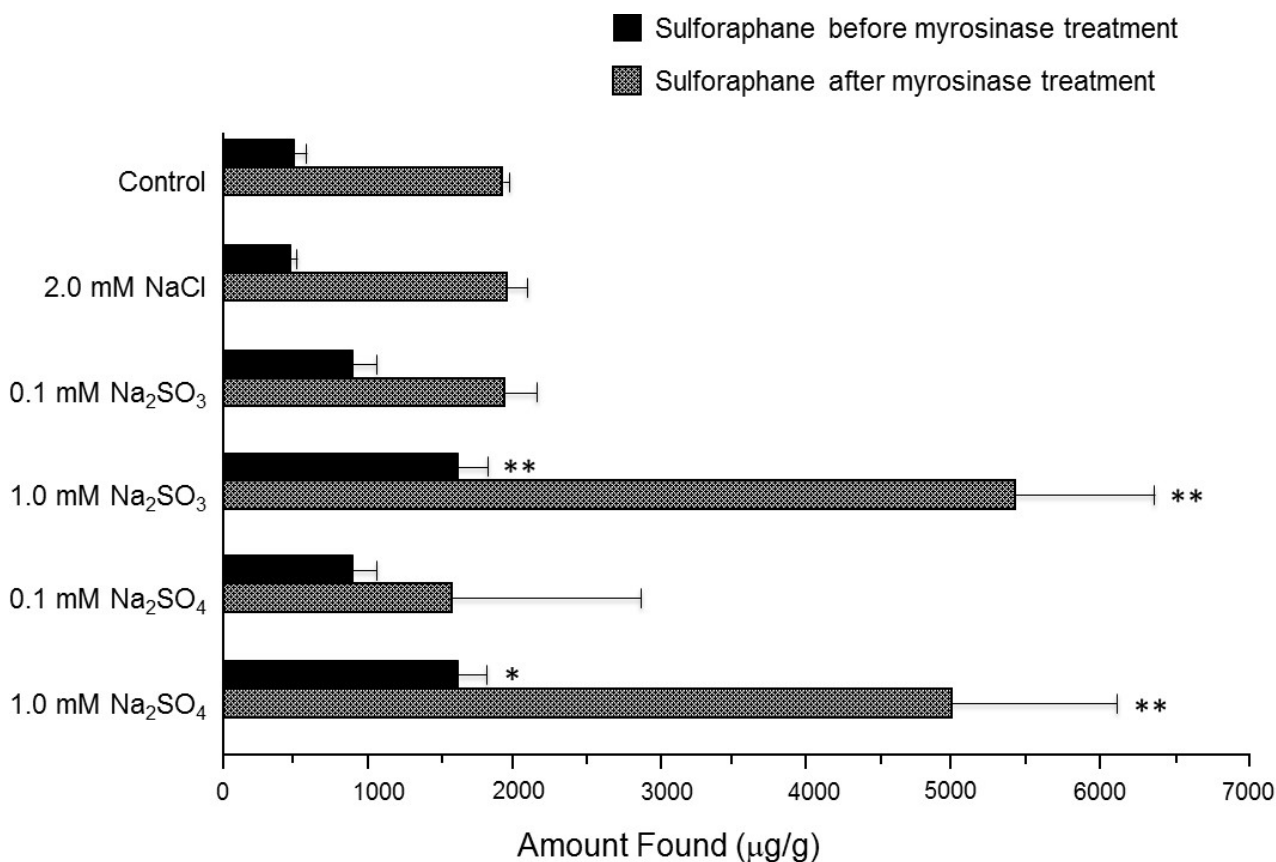


Fig.VI. Analytical results of sulforaphane (Compound VIII) in the chloroform extracts from sprout samples before and after myrosinase treatment. Values are means \pm SD (n = 3). *: p < 0.05, **: p < 0.01.

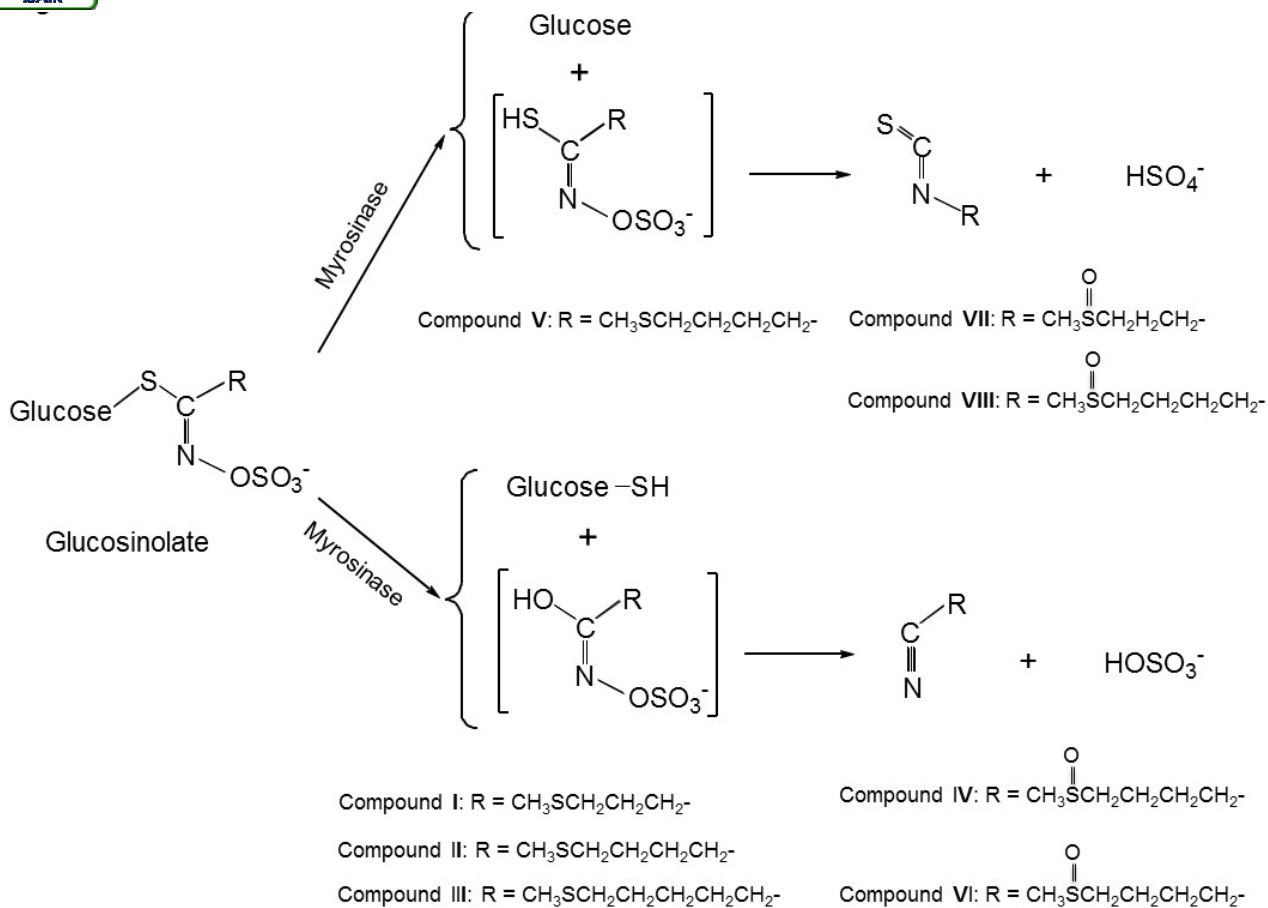


Fig.VII. Proposed formation pathways of isothiocyanates and nitriles from corresponding glucosinolate