MAXIMIZING SULFORAPHANE DELIVERY AND SENSORY ACCEPTABILITY OF A NOVEL SOY-TOMATO-BROCCOLI SPROUT BEVERAGE

A Thesis
Presented in Partial Fulfillment of the Requirements for the Degree Master of Sciences in the Graduate School of The Ohio State University

By
Ryan T. Riddle, B.S.
Graduate Program in Food Science and Technology
The Ohio State University
2011

Master’s Examination Committee:
Professor Steven Schwartz, Adviser
Professor Luis Rodriguez-Saona
Professor Sheryll Barringer
Copyright by

Ryan Thomas Riddle

2011
ABSTRACT

Recent epidemiological studies have suggested that consumption of cruciferous vegetables like broccoli, cabbage, and cauliflower may convey potent cancer preventative effects, and subsequent *in vivo* and *in vitro* studies have linked this anticancer activity to a group of compounds that cruciferous vegetables contain called isothiocyanates. Furthermore, additional research has indicated that consumption of cruciferous vegetables and tomatoes together could provide an added preventative effect against cancer. As part of an ongoing effort to develop bioactive-enhanced functional foods designed to provide cancer preventative effects, we sought to find a way to incorporate broccoli sprouts, which contain high levels of isothiocyanates, into a soy-tomato beverage previously developed by our group. The goal of this project was to develop a formulation for this new soy-tomato-broccoli sprout beverage that maximizes potential cancer preventative effects while retaining sensory acceptability.

To accomplish this goal, several prototypes of a freeze-dried broccoli sprout powder were developed based on recent research that indicates processing methods may greatly affect the healthful effects and sensory attributes of cruciferous vegetables. Subsequently, sensory tests were conducted to determine how these processing methods affect the sensory properties of the powders when they are added to our soy-tomato juice, as well as the maximum amount of broccoli sprout powder that can be added to the juice before sensory acceptability is significantly hampered. The results of these studies have
indicated that a freeze-dried powder consisting of steamed broccoli sprouts and raw daikon radish sprouts provides the desired attributes to the beverage and that a concentration of about 0.5% is appropriate. Furthermore, our data suggests that the superiority of this formulation may be due to a delayed release of isothiocyanates that minimizes the pungent aroma of the broccoli sprouts while retaining their healthful effects. These results help to further the goal of developing scientifically designed functional foods that will serve to prevent cancer in future at-risk patients.
ACKNOWLEDGMENTS

Thank you to Dr. Steven Schwartz and everyone in the Schwartz lab at The Ohio State University, whose guidance and assistance in my project was truly indispensible.

Thank you to the faculty and staff at the OSU Department of Food Science, including Melody Leidheiser for her assistance with administration of sensory studies, Paul Courtwright and Gary Wenneker for their assistance in the pilot plant, and Dr. Kyle Kent for his scientific and philosophical advice.

Thank you to my parents, Tom and Colleen Riddle, who only ever expected that I follow my dreams.

Thank you to Carl Sagan, Bill Nye, Richard Feynman, Alton Brown, Richard Dawkins, and Neil DeGrasse Tyson for inspiring me with the beauty of science.
VITA

October 26, 1987 ................................................................. Born, Houston, TX

2002-2006 ................................................................. Spring High School, Spring, TX

2006-2010 ................................................................. BS Honors Biochemistry (Dean’s Scholars),

The University of Texas at Austin

2010-Present ................................................................. Graduate Research Associate,

The Ohio State University

FIELDS OF STUDY

Major Field: Food Science and Technology
TABLE OF CONTENTS

Page

Abstract ................................................................................................................................. ii
Acknowledgments .............................................................................................................. iv
Vita ....................................................................................................................................... v
Table of Contents ............................................................................................................... vi
List of Figures ..................................................................................................................... ix
List of Tables ....................................................................................................................... xii
List of Abbreviations ......................................................................................................... xiii
Chapter 1: Background ........................................................................................................ 1
  1.1 Introduction .................................................................................................................. 1
  1.2 Chemistry of GLUs and ITCs ..................................................................................... 6
  1.3 Quantitative analysis of isothiocyanates .................................................................. 9
    1.3.1 Gas chromatography ............................................................................................. 9
    1.3.2 Liquid chromatography ....................................................................................... 10
      1.3.2.1 High performance liquid chromatography-photo diode array (HPLC-PDA) .............. 10
      1.3.2.2 Conjugation with vicinal dithiols ................................................................. 11
      1.3.2.3 Conjugation with 2-mercaptoethanol ......................................................... 12
    1.3.3 Summary ............................................................................................................... 13
  1.4 Isothiocyanates, cruciferous vegetables, and their effects on health ...................... 14
  1.5 Effects of processing cruciferous vegetables on health and sensory attributes .......... 18
  1.6 Effects of carotenoids, isoflavones, and isothiocyanates on health ....................... 23
  1.7 Research plan .............................................................................................................. 25
Chapter 2: Maximizing sulforaphane delivery and sensory acceptability of a novel soy-tomato-broccoli sprout beverage

2.1 Abstract .................................................................................................................. 28

2.2 Introduction .............................................................................................................. 30

2.2.1 Maximizing sulforaphane delivery via freeze-dried broccoli sprout powders ................................................................. 31

2.2.2 Determining sensory acceptability of soy-tomato-broccoli sprout beverages ................................................................................. 38

2.3 Materials and methods .......................................................................................... 41

2.3.1 Effects of 60°C pretreatments on sulforaphane formation in broccoli sprouts .................................................................................. 41

2.3.2 Utilization of raw daikon radish sprouts as a source of myrosinase .......................................................................................... 41

2.3.3 Extraction and quantification ITCs in broccoli sprout homogenates ......................................................................................... 42

2.3.4 Spike-recovery tests for reproducibility of quantifying ITCs by 2-mercaptoethanol conjugation .......................................................... 43

2.3.5 Production of freeze-dried broccoli sprout powders ......................................................................................................................... 44

2.3.5.1 Heat treatments ......................................................................................................................... 44

2.3.5.2 Flash freezing and freeze-drying ........................................................................................ 45

2.3.5.3 Grinding freeze-dried sprouts into powders ........................................................................... 45

2.3.6 Measuring formation of sulforaphane in soy-tomato juice after the addition of broccoli sprout powders ........................................... 46

2.3.7 Analysis of GLUs in broccoli sprout powders ................................................................................................. 47

2.3.8 Canning of soy-tomato juice .................................................................................... 48

2.3.9 Analysis of lycopene in soy-tomato juices .................................................................. 49

2.3.10 Analysis of isoflavones in soy-tomato juices and soy isoflavone concentrate ................................................................................... 50

2.3.11 Sensory studies ...................................................................................................... 51

2.3.11.1 Approval for institutional review board (IRB) exemption ................................................................................................. 51
2.3.11.2 Recruitment ................................................................. 51
2.3.11.3 Administration of sensory experiments ....................... 52

2.4 Results and discussion ........................................................................ 52

2.4.1 Analysis of ITCs by conjugation to 2-mercaptoethanol and
detection with HPLC-PDA ................................................................. 52
2.4.2 Effects of 60°C pretreatment on sulforaphane formation in
broccoli sprouts .................................................................................. 57
2.4.3 Utilization of raw daikon radish sprouts as a food grade source
of myrosinase ...................................................................................... 58
2.4.4 Formation of sulforaphane in soy-tomato juice after addition of
broccoli sprout powders ................................................................. 61
2.4.5 Sensory properties of various broccoli sprout powders in soy-
tomato juice ...................................................................................... 64
2.4.6 Sensory effects of varying concentration of SDP in soy-tomato
juice ...................................................................................................... 69

2.5 Conclusions .......................................................................................... 76

References ................................................................................................. 79

Appendix A: Sensory evaluation ballot provided to participants ............ 93
Appendix B: Written consent form provided to sensory participants .......... 96
Appendix C: Approval letters for exemption from IRB review ................. 101
Appendix D: Recruitment letter for sensory studies ................................. 104
Appendix E: Standard curves .................................................................. 107
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure Name</th>
<th>Page Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1 General structure of glucosinolates and isothiocyanates</td>
<td>6</td>
</tr>
<tr>
<td>1.2 Conversion of glucoraphanin to sulforaphane, an example of how myrosinase catalyzes the conversion of glucosinolates to isothiocyanates</td>
<td>8</td>
</tr>
<tr>
<td>1.3 Formation of sulforaphane nitrile by ESP</td>
<td>20</td>
</tr>
<tr>
<td>1.4 Bioavailability of isothiocyanates and glucosinolates in cooked and raw broccoli. Adapted from Shapiro and others (2001)</td>
<td>22</td>
</tr>
<tr>
<td>2.1 Formation of sulforaphane nitrile by ESP</td>
<td>32</td>
</tr>
<tr>
<td>2.2 Bioavailability of isothiocyanates and glucosinolates in cooked and raw broccoli. Adapted from Shapiro and others (2001)</td>
<td>36</td>
</tr>
<tr>
<td>2.3 Comparison of UV spectra of sulforaphane and sulforaphane-mercaptoethanol conjugate</td>
<td>55</td>
</tr>
<tr>
<td>2.4 Chromatogram showing separation of sulforaphane, erucin, and iberin conjugated to 2-mercaptoethanol in broccoli sprout homogenate</td>
<td>56</td>
</tr>
<tr>
<td>2.5 Sulforaphane concentration in raw and 60°C pretreated broccoli sprout homogenates</td>
<td>58</td>
</tr>
<tr>
<td>2.6 Concentration of sulforaphane in steamed broccoli sprout homogenates incubated with purified myrosinase at various conditions</td>
<td>60</td>
</tr>
</tbody>
</table>
2.7 Concentration of sulforaphane in steamed broccoli sprout homogenates following incubation with raw daikon sprouts and purified myrosinase, as compared to that in raw broccoli sprout homogenate.................................61

2.8 Concentration of sulforaphane in soy-tomato juice at various times following the addition of freeze-dried broccoli sprout powders..............................62

2.9 Overall acceptability of various freeze-dried broccoli sprout powders in soy-tomato juice.........................................................................................................................65

2.10 Just About Right (JAR) scores for amount of pungency and broccoli flavor of various freeze-dried broccoli sprout powders in soy-tomato juice.............67

2.11 Rank total of various freeze-dried broccoli sprout powders in soy-tomato juice .................................................................................................................................69

2.12 Concentrations of glycitin, daidzein, and genistein in soy-tomato juice........71

2.13 Concentrations of daidzin and genistin in soy-tomato juice.....................71

2.14 Average overall acceptability of freeze-dried steamed broccoli sprout plus raw daikon sprout powder (SDP) at various concentrations in soy-tomato juice.....72

2.15 Just About Right (JAR) scores for amount of pungency and broccoli flavor in soy-tomato juice with freeze-dried steamed broccoli sprout and raw daikon radish sprout powder (SDP) at various concentrations........................................73

2.16 Rank total of freeze-dried steamed broccoli sprout plus raw daikon sprout powder (SDP) at various concentrations in soy-tomato juice............................75

E.1 Standard curves of sulforaphane-mercaptoethanol conjugate used to quantify sulforaphane concentration in broccoli sprout homogenates and soy-tomato juice.........................................................................................................................108

E.2 Standard curves of iberin- and erucin-mercaptoethanol conjugates used to quantify iberin and erucin concentrations in broccoli sprout homogenates ....109
E.3 Standard curves of allyl ITC- and phenethyl ITC-mercaptoethanol conjugates used to quantify allyl ITC and phenethyl ITC in spike-recovery experiment in broccoli sprout homogenates ..........................................................110

E.4 Standard curves of daidzin, genistin, and glycitin used to quantify concentrations of these isoflavones in soy-tomato juice .................................................................111

E.5 Standard curves for daidzein and genistein used to quantify concentrations of these isoflavones in soy-tomato juice .................................................................112

E.6 Standard curve for all-trans-lycopene used to quantify lycopene concentration in soy-tomato juice .................................................................112
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table Name</th>
<th>Page Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1  Summary of effects of sulforaphane on enzymes and transcription factors (Clarke, Dashwood, and Ho 2008)</td>
<td>16</td>
</tr>
<tr>
<td>2.1  Recovery rates of various ITCs when analyzed by the mercaptoethanol conjugation method</td>
<td>57</td>
</tr>
<tr>
<td>2.2  Concentrations of glucoraphanin, glucoerucin, and glucoiberin in freeze-dried broccoli sprout powders</td>
<td>64</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Meaning</td>
</tr>
<tr>
<td>--------------</td>
<td>---------</td>
</tr>
<tr>
<td>60P</td>
<td>60°C pretreated freeze-dried broccoli sprout powder</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ESP</td>
<td>Epithiospecifier protein</td>
</tr>
<tr>
<td>FID</td>
<td>Flame ionization detection</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>GLU</td>
<td>Glucosinolate</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>HSD</td>
<td>Honestly significant difference</td>
</tr>
<tr>
<td>IRB</td>
<td>Institutional review board</td>
</tr>
<tr>
<td>ITC</td>
<td>Isothiocyanate</td>
</tr>
<tr>
<td>JAR</td>
<td>Just about right</td>
</tr>
<tr>
<td>LCMS</td>
<td>Liquid chromatography-mass spectrometry</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MTBE</td>
<td>Methyl tert-butyl ether</td>
</tr>
<tr>
<td>PDA</td>
<td>Photo diode array</td>
</tr>
<tr>
<td>RP</td>
<td>Raw freeze-dried broccoli sprout powder</td>
</tr>
<tr>
<td>SDP</td>
<td>90% steamed broccoli sprout plus 10% raw daikon sprout freeze-dried powder</td>
</tr>
<tr>
<td>SP</td>
<td>Steamed freeze-dried broccoli sprout powder</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet light</td>
</tr>
</tbody>
</table>
CHAPTER 1: BACKGROUND

1.1 Introduction

Cruciferous vegetables like broccoli, cabbage, and cauliflower contain large quantities of molecules called glucosinolates (GLUs). When the cells of these vegetables are breached, GLUs interact with an enzyme called myrosinase, which catalyzes their conversion to isothiocyanates (ITCs). Recently, ITCs have garnered a great deal of interest for their anticancer effects. Epidemiological studies have shown a particularly promising link between consumption of cruciferous vegetables and reduced rates of cancer (Michaud and others 1999), and subsequent in vivo and in vitro studies have linked this anticancer activity to the ITCs that cruciferous vegetables contain (Gamet-Payrastre and others 2000; Hecht 2000; Conaway, Yang, and Chung 2002; Solt and others 2003).

One ITC in particular, sulforaphane, has shown extremely strong anticancer activity. Sulforaphane has been shown to slow cancer cell growth in vitro and tumor growth in vivo, primarily by upregulating phase II detoxification enzymes like quinone reductase and downregulating phase I oxidative enzymes like cytochrome P450 (Bonneseen, Eggleston, and Hayes 2001; Ritz, Wan, and Diaz-Sanchez 2007; Harris and Jeffery 2008). Since sulforaphane is especially abundant in broccoli sprouts (Fahey,
Zhang, and Talalay 1997; Tian, Rosselot, and Schwartz 2005), there is great interest in using broccoli sprouts to prevent cancer occurrence via a functional food.

Previously, our group developed a functional beverage consisting of tomato juice and soy isoflavone extract (Bohn and others 2005). This beverage has high levels of lycopene and isoflavones, which have been shown to have anticancer properties. A recent development suggesting that the consumption of broccoli and tomato together may convey additional cancer preventative effects has provided the impetus for the development of a soy-tomato-broccoli sprout beverage that utilizes the anticancer properties of sulforaphane in addition to those of lycopene and isoflavones (Canene-Adams and others 2007).

The effectiveness of a functional beverage should be measured not only by its ability to have positive health effects when consumed, but also by the acceptability of its sensory characteristics. When developing the soy-tomato beverage, our group added a small amount of olive oil to enhance lycopene bioavailability and utilized an extract of soy that did not adversely affect the flavor of the juice. The incorporation of broccoli into the beverage provides its own similar challenges. The purpose of the project discussed in the following chapters was to determine the formulation of this new soy-tomato-broccoli sprout beverage that provides the best compromise between the hypothesized cancer preventative potential and sensory acceptability.

One challenge associated with incorporating broccoli sprouts into a tomato-based beverage is that GLUs and ITCs are thermolabile (Engel and others 2002; Verkerk and
Heat inactivates the myrosinase enzyme, which slows or halts the formation of ITCs during processing. In addition, relatively severe heat treatments, such as those used during the canning of tomato juice, can degrade GLUs, ITCs, and other sulfur containing molecules into odious volatile compounds (Engel and others 2002; Van Eylen and others 2007). To circumvent these issues, we chose to develop freeze-dried broccoli sprout powders, processed without thermally intensive methods, which will be mixed into the tomato juice immediately before consumption. These prototypes vary in the way the broccoli sprouts are processed before freeze-drying. These methods were chosen based on research suggesting that processing methods may affect the formation of sulforaphane and thus the cancer preventative potential of the broccoli sprouts (Howard and others 1997; Matusheski, Juvik, and Jeffery 2004; Matusheski and others 2006; Van Eylen and others 2007; Jones and others 2010).

Matusheski and others (2006) previously discovered that an epithiospecifier protein (ESP) present in broccoli and broccoli sprouts can decrease sulforaphane formation in broccoli by converting its precursor into sulforaphane nitrile, which has not been shown to have any anticancer activity (Matusheski and Jeffery 2001). Thus, the presence of ESP in broccoli sprouts could decrease their cancer preventative potential. In addition, the gut flora of the animal intestine has been shown to exhibit myrosinase activity, meaning that consumption of GLUs results in absorption of ITCs (Getahun and Chung 1999; Lai and others 2008). Based on these results, it is reasonable to hypothesize that heating broccoli to inactivate ESP (and myrosinase) could increase sulforaphane absorption; however, subsequent research has shown that consumption of raw cruciferous vegetables containing active myrosinase results in greater ITC absorption than
consumption of GLUs alone (Conaway and others 2000; Shapiro and others 2001; Keck, Qiao, and Jeffery 2003; Zhu and others 2010). The way broccoli sprouts are heat-treated has therefore been shown to have a substantial effect on the bioavailability of the sulforaphane and thus its theoretical cancer preventative potential.

Interestingly, Matusheski, Juvik, and Jeffery (2004) found that heating broccoli sprouts to 60ºC for 10 minutes can inactivate ESP without inactivating myrosinase, increasing the amount of sulforaphane formed in vitro by a factor of 10. In addition, daikon radish sprouts have been shown to be an excellent source of myrosinase without any detectable presence of ESP (Matusheski and others 2006). This has led our group to hypothesize that the best methods for heat treating broccoli sprouts, in terms of maximizing their cancer preventative potential, may be either to heat them to 60º to inactivate ESP or to steam them and combine them with daikon radish sprouts, which contribute natural myrosinase.

Based on these findings, we developed four prototypes of freeze-dried broccoli sprout powder that can be mixed with our soy-tomato juice to create new soy-tomato-broccoli sprout beverages: 1) raw broccoli sprouts (RP), 2) steamed broccoli sprouts (SP), 3) 60ºC pretreated broccoli sprouts (60P), and 4) 90% steamed broccoli sprouts with 10% raw daikon radish sprouts (SDP). We then performed sensory experiments to determine which of these powders provides the beverage with the best compromise between theoretical cancer preventative potential and sensory acceptability and what concentration of broccoli sprout powder is acceptable to add to the juice before the sensory acceptability is significantly hampered.
Since heat treatments have been shown to affect the activity of myrosinase and ESP in broccoli sprouts, we hypothesized that our various freeze-dried broccoli sprout powders would contribute varying concentrations of sulforaphane to our soy-tomato juice. Specifically, we predicted that 60P and SDP would contribute the most sulforaphane due to the presence of active myrosinase and absence of ESP. We also predicted that RP would contribute more sulforaphane to the juice than SP due to the presence of myrosinase.

Furthermore, since ITCs contribute pungency to foods and heat treatments produce volatile compounds such as sulfides, we also hypothesized that the various freeze-dried powders would provide varying sensory properties to the soy-tomato juice. Specifically, we predicted that SP would have the most favorable sensory properties due to the lack of myrosinase and presence of small amounts of cooked broccoli flavor. We hypothesized that 60P and SDP would contribute the most pungency to the juice and would therefore be less favorable. We were interested in determining the extent of the differences in sensory properties of our various soy-tomato-broccoli sprout beverages.

In the following chapters, the development of these prototypes and the two sensory experiments performed on them are described. Before going into detail on these endeavors, however, it is important to recognize the established scientific knowledge regarding isothiocyanates, cruciferous vegetables, and how processing effects their sensory and healthful properties. The sections that follow in the first chapter represent a thorough background of the scientific literature used to complete this project.
1.2 Chemistry of GLUs and ITCs

ITCs exist in cruciferous vegetables as their glycosylated precursors, GLUs. In general, GLUs are $\beta$-thioglucoside $N$-hydroxysulfates (Figure 1.1) (Fahey, Zalcmann, and Talalay 2001). GLUs differ in the structure of their side chains, which can vary greatly. Most GLU side chains are straight or branched carbon chains, but some contain sulfur groups, aromatic groups, and other structures (Fahey, Zalcmann, and Talalay 2001). GLUs are found primarily in cruciferous vegetables but can be found in some other plant families as well (Fenwick and Heaney 1983). The structures of GLUs found in cruciferous vegetables vary greatly from species to species; in fact, the structures of GLUs in plants can be used to determine their evolutionary history (Mithen, Bennett, and Marquez 2010).

![Figure 1.1 General structure of glucosinolates (left) and isothiocyanates (right).](image)

The function of GLUs in plants is primarily to serve as deterrents to herbivores. When the cells of cruciferous vegetables are breached, GLUs mix with the enzyme myrosinase, which is compartmentalized in myrosin cells (Mortazavi and others 2008). Myrosinase catalyzes the conversion of GLUs to ITCs. ITCs do not normally form in plant cells without disruption of the cellular matrix (Fenwick and Heaney 1983). ITCs are
toxic to many animals and thus serve to protect the plant from herbivores (Hopkins, Van Dam, and Van Loon 2009). Some animals may even sequester GLUs for their own defense against other animals (Hopkins, Van Dam, and Van Loon 2009). ITCs also have fungicidal and bactericidal properties (Fahey, Zalcmann, and Talalay 2001).

Myrosinase is a $\beta$-thioglucosidase and thus deglycosylates GLUs to form intermediate compounds that undergo a Lossen-type rearrangement to form ITCs (Fenwick and Heaney 1983) (Figure 1.2). Since their side chains are unaffected by the reaction, each GLU has a corresponding ITC containing the same side chain with a characteristic $N=C=S$ moiety. Myrosinase requires ascorbic acid and ferrous iron to act on GLUs (Shikita and others 1999). Myrosinase has greatest activity at pH near 6.25, but it is still active at very acidic pH (Ludikhuyze, Rodrigo, and Hendrickx 2000). Myrosinase can be found in some fungi as well as in gut microbiota of some mammals, including humans (Fenwick and Heaney 1983; Getahun and Chung 1999).
The primary GLUs found in broccoli are glucoraphanin (the precursor to sulforaphane), glucoerucin, and glucoiberin. Broccoli also contains 4-methoxyglucobrassicin, neoglucobrassicin, and progoitrin in lesser quantities (Tian, Rosselot, and Schwartz 2005). Glucoraphanin is found in the highest concentrations in broccoli florets, as compared to the stalks and leaves (Campas-Baypoli and others 2010). Based on the current body of evidence, broccoli appears to contain higher concentrations of glucoraphanin than any other fruit or vegetable (Kushad and others 1999), and broccoli sprouts contain even greater concentrations of glucoraphanin than mature broccoli (Fahey, Zhang, and Talalay 1997; Tian, Rosselot, and Schwartz 2005). Studies on the
effects of broccoli genotype and growing conditions have led to the development of cultivars of broccoli sprouts that contain extremely high concentrations of glucoraphanin (Pereira and others 2002; Farnham, Stephenson, and Fahey 2005).

ITCs are electrophilic and react readily with nucleophiles such as amines, amino acids, proteins, and sulfites (Zhang and others 1996; Kim and others 2008; Zhu and others 2010). They are very soluble in organic solvents but only sparingly soluble in water (Depree and Savage 2002). Due to their high reactivity with nucleophiles, it is common to derivatize them before analysis, which is discussed in the following section (Zhang and others 1992; Zhang and others 1996). ITCs are somewhat thermolabile (Van Eylen and others 2007), but they may be stabilized somewhat by citric acid (Depree and Savage 2002).

1.3 Quantitative analysis of isothiocyanates

Accurate, quantitative analytical methods for ITCs and GLUs are imperative for research involving cruciferous vegetables. A wide variety of analytical methods have been used to quantify GLUs, ITCs, and their biological metabolites. This section summarizes the most common methods used, their advantages and disadvantages, and how we chose to analyze ITCs and GLUs during the development of our soy-tomato-broccoli sprout beverage.

1.3.1 Gas chromatography

One common method for analyzing GLUs and their hydrolysis products is gas chromatography (GC), coupled with either flame ionization detection (FID) or mass spectrometry (MS). The relatively high volatility of ITCs allows them to be effectively separated by GC (Kore, Spencer, and Wallig 1993), but GLUs must be desulfated or
derivatized before separation by GC due to their ionic nature (Smiechowska, Bartoszek, and Namiesnik 2010). GC coupled with MS provides a useful tool for identifying a wide variety of hydrolysis products of GLUs, including ITCs and nitriles (Chiang, Pusateri, and Leitz 1998; Al-Gendy and Lockwood 2003). GC is useful for qualitative analysis of GLUs and ITCs due to its low running cost and analysis time per sample; unfortunately, the thermolability of GLUs and ITCs results in their degradation during separation and detection in GC (Smiechowska, Bartoszek, and Namiesnik 2010). Thus, GC is a useful method for identification of GLUs and their breakdown products, but it is not an ideal method for quantification.

1.3.2 Liquid chromatography

1.3.2.1 High performance liquid chromatography-photo diode array (HPLC-PDA)

HPLC has an advantage over GC in that it allows GLUs and ITCs to be analyzed without thermally intensive methods that would degrade them, reducing accuracy of quantification. As a result, the recovery of ITCs and GLUs by HPLC analysis is high (Campos-Baypoli and others 2010). Preparative HPLC is a very useful method for purifying GLUs and ITCs since it does not degrade them (Matusheski and others 2001). GLUs can be analyzed directly by liquid chromatography using anion exchange columns or desulfated and analyzed by reverse-phase HPLC (Szmigielska and Schoenau 2000). Unfortunately, ITCs are difficult to quantify directly by HPLC-PDA due to their low molar absorptivity in the UV region. As a result, samples must be concentrated heavily to allow for accurate quantification of ITCs. Solid phase extraction, for example, can be used before HPLC analysis to increase the sensitivity of ITC quantification (Bertelli and others 1998). Even after concentration, ITCs present in low concentrations are difficult to
quantify directly by HPLC. As a result, separation of intact ITCs by HPLC is useful for quantifying ITCs present in high concentrations, such as sulforaphane in broccoli, but accurate quantification of minor ITCs requires that they be derivatized before analysis.

1.3.2.2 Conjugation with vicinal dithiols

The most common method of derivatizing ITCs to increase detection limits is to allow them to undergo cyclocondensation with vicinal dithiols, such as 1,2-benzenedithiol. This method was first described by the Talalay group at Johns Hopkins University (Zhang and others 1992) and has been used in numerous subsequent studies to quantify total ITCs in food and biological samples (Prestera and others 1996; Shapiro and others 1998; Getahun and Chung 1999; Conaway and others 2000; Shapiro and others 2001; Zhang 2010). The reaction of ITCs with vicinal dithiols such as 1,2-benzenedithiol forms a cyclic compound containing the carbon and sulfur atoms from the NCS group of the ITC as well as a primary amine containing the side chain of the ITC. The resulting cyclic compound has a dramatically higher molar absorptivity, and total ITC concentration can be quantified using a spectrophotometer at 365 nm (Zhang and others 1992). The sensitivity of the method can be further improved by analyzing samples by HPLC-PDA (Zhang and others 1996).

The primary drawback of the cyclocondensation method is that ITC side chains are not incorporated into the cyclic compound, meaning that all ITCs produce the same end product. Quantification of individual ITCs is thus not possible using this method unless the ITCs are separated first (Prestera and others 1996). Nevertheless, the cyclocondensation method is useful for measuring total ITCs in food samples and provides a more sensitive method for measuring myrosinase activity in samples than
measuring the formation of glucose, which is produced when myrosinase acts on GLUs (Zhang and others 1992).

1.3.2.3 Conjugation with 2-mercaptoethanol

Another method for derivatizing ITCs before quantification by HPLC-PDA involves reacting ITCs with 2-mercaptoethanol. Like derivatization with vicinal dithiols, conjugation with 2-mercaptoethanol greatly increases the molar absorptivity of ITCs, but unlike the cyclocondensation reaction, derivatizing ITCs to 2-mercaptoethanol preserves the unique side chains of the ITCs (Vermeulen and others 2006). This means that ITCs can be separated by reverse-phase HPLC after 2-mercaptoethanol derivatization, allowing for the quantification of individual ITCs in both food and biological samples (Vermeulen 2008). The primary advantage of the cyclocondensation method over derivatization with 2-mercaptoethanol is that the molar absorptivity of ITC cyclocondensation products is somewhat higher than ITC-mercaptoethanol conjugates, but the ability of 2-mercaptoethanol conjugation to allow quantification of individual ITCs makes it a much more useful method when total ITC quantification is insufficient.

1.3.2.4 HPLC-mass spectrometry (MS)

The advantage of utilizing HPLC to analyze GLUs and ITCs is that they can be effectively separated without using thermally intensive methods that could result in their degradation. Unfortunately, the charged nature of GLUs and the low molar absorptivity of ITCs hinder HPLC-PDA from being the most effective tool for quantifying GLUs and ITCs in food and biological samples, especially when they are present at low concentrations. Fortunately, the high sensitivity of MS makes liquid chromatography-mass spectrometry (LCMS) an extremely powerful tool for quantifying GLUs and ITCs
without the need for derivatization. LCMS has been used to quantify the spectrum of GLUs in food samples as well as their degradation products, including ITCs and nitriles (Song and others 2005; Tian, Rosselot, and Schwartz 2005). In addition, the high sensitivity of LCMS make it the best tool for quantifying concentrations of GLU degradation products and ITC metabolites in biological samples since ITC metabolites are often present in very low concentrations (Vermeulen and others 2003; Song and others 2005; Egner and others 2008). The major drawback of LCMS is that it is relatively expensive compared to other methods, and as a result it is not always readily available.

1.3.3 Summary

Several distinct analytical methods have been utilized to quantify GLUs and ITCs in both plant and biological samples. GC and GC-MS are sensitive and rapid but can result in degradation of ITCs, reducing accuracy. HPLC coupled with PDA is useful for samples in which ITC concentrations are high, but without conjugating ITCs to increase the sensitivity of the analysis, low concentrations of ITCs cannot be quantified. Cyclocondensation with vicinal dithiols has been widely used, but it only allows for quantification of total ITCs unless they are separated first, making individual identification of ITCs laborious. Conjugation of ITCs to mercaptoethanol, conversely, is rapid and allows for separation and identification of ITCs by HPLC-PDA, increasing the sensitivity over analyzing unaltered ITCs by an order of magnitude. Finally, HPLC coupled with MS is a sensitive, powerful method for analyzing GLUs, ITCs, and their biological metabolites, but it is more expensive and less accessible than other methods. For these reasons, we chose primarily to utilize conjugation with mercaptoethanol followed by analysis by HPLC-PDA to measure ITCs in plant samples.
1.4 Isothiocyanates, cruciferous vegetables, and their effects on health

The healthful effects of cruciferous vegetables, GLUs, and ITCs have been heavily researched. Epidemiological studies have suggested that consumption of cruciferous vegetables may convey a wide variety of healthful effects. Studies have linked consumption of cruciferous vegetables to reduced rates of lung cancer (London and others 2000) and bladder cancer (Michaud and others 1999), suggesting that they may have higher cancer preventative effects than other types of fruits and vegetables. Other studies have shown a link between cruciferous vegetable consumption and increased longevity in patients suffering from bladder cancer (Tang and others 2010) as well as an increase in overall longevity and a decrease in cardiovascular disease risk (Zhang and others 2011). These studies have warranted further studies on the possible mechanisms responsible for the healthful effects of cruciferous vegetables.

A multitude of *in vitro* and *in vivo* studies have linked the cancer preventative effects of cruciferous vegetables to the GLUs and ITCs that they contain. *In vitro*, ITCs such as sulforaphane, erucin, benzyl ITC, allyl ITC, and phenethyl ITC as well as cruciferous vegetable extracts have been shown to induce apoptosis and inhibit the growth of cancer cells of the colon (Gamet-Payrastre and others 2000; Bonnesen, Eggleston, and Hayes 2001), liver (Lamy and others 2008), breast (W. Wang and others 2005; Meeran, Patel, and Tollefsbol 2010), and lung (Yang and others 2005; Ritz, Wan, and Diaz-Sanchez 2007; Melchini and others 2009; Wu and others 2010) of human and rodent origin. In addition *in vivo* studies have shown similar effects on the progression of bladder (Bhattacharya and others 2010), prostate (Canene-Adams and others 2007; Keum and others 2009; Liu and others 2009; Powolny and others 2011), lung (Conaway and
others 2005), breast (Fahey, Zhang, and Talalay 1997), skin (Gills and others 2006; Lee and others 2011), colon (Keck, Qiao, and Jeffery 2003), and oral cancers (Solt and others 2003), primarily in rodent models. In addition to their anticancer effects, ITCs have been shown to have positive effects on cholesterol metabolism (Rodríguez-Cantú and others 2011), systemic genotoxicity (Shaughnessy and others 2011), and neuroprotective effects (Ping and others 2010). ITCs can also be utilized for their antimicrobial effects (Kim and others 2008; Vega-Lugo and Lim 2009); they have been shown to inhibit the growth of *Escherichia coli* O157:H7 (Chacon, Buffo, and Holley 2006), *Salmonella* spp., and *Helicobacter pylori* (Fahey and others 2002; Shin, Masuda, and Naohide 2004). There are several thorough review articles that summarize these *in vitro* and *in vivo* studies (Conaway, Yang, and Chung 2002; Higdon and others 2007; Mortazavi and others 2008; Melchini and Traka 2010; Cavell and others 2011).

There appear to be numerous mechanisms through which ITCs inhibit the growth of cancerous cells. ITCs downregulate many oxidative enzymes such as various cytochrome P450 enzymes and upregulate many detoxification enzymes such as quinone reductase (Bonnesen, Eggleston, and Hayes 2001). ITCs have also been shown to inhibit cell cycle progression and induce apoptosis in a wide variety of cell lines, and they have been shown to inhibit angiogenesis and metastasis in *in vitro* and *in vivo* studies. Cavell and others (2011) have written a thorough review of the mechanisms by which ITCs inhibit growth of cancerous cells, and Clarke, Dashwood, and Ho (2008) have written an excellent summary of the effects of sulforaphane on various enzymes and transcription factors involved in cancer progression. Table 1.1 summarizes these findings.
Recent studies have investigated the mechanisms by which GLUs and ITCs are absorbed in the body. Although a small proportion of GLUs may be absorbed intact, they are primarily absorbed after being converted to ITCs (Bheemreddy and Jeffery 2007). Conversion from GLUs to ITCs in the body can occur as the result of active myrosinase present in the food or from the myrosinase activity present in the human gut microbiota (Getahun and Chung 1999; Lai, Miller, and Jeffery 2010). After ITCs are absorbed in the intestine, they are primarily metabolized through the mercapturic acid pathway, first conjugated to glutathione (Egner and others 2008). The most abundant metabolite of ITCs observed in urine is the N-acetylcysteine conjugate (Shapiro and others 1998; Vermeulen, Van Rooijen, and Vaes 2003; Egner and others 2008).
Several studies have tested the bioavailability of GLUs and ITCs with similar results; the quantity of ITC metabolites recovered in urine after cooked cruciferous vegetables or GLUs are consumed is approximately 5-15%, whereas consumption of ITCs results in 70-90% recovery of metabolites (Conaway and others 2000; Shapiro and others 2001; Bheemreddy and Jeffery 2007; Hanlon and others 2008; Vermeulen and others 2008). Furthermore, consumption of raw cruciferous vegetables containing active myrosinase results in the recovery of approximately 50% of GLU metabolites in the urine, indicating that delivery of ITCs may be improved by a factor of up to 10 if myrosinase activity in the food is left intact. Studies performed in conjunction with our laboratory have shown that consumption of raw broccoli sprouts results in absorption of approximately four times more sulforaphane and erucin in urine than consumption of broccoli supplements lacking myrosinase activity (Clarke and others 2011a). A subsequent study showed that consumption of the raw broccoli sprouts sped the absorption of sulforaphane and resulted in decreased histone deacetylase activity as compared to consumption of the broccoli supplements, even though the dose of GLUs was the same (Clarke and others 2011b).

Several studies have indicated that consumption of whole cruciferous vegetables containing enhanced levels of bioactives may result in greater delivery of ITCs and/or a greater anticancer effect than consumption of purified phytochemicals (Keck, Qiao, and Jeffery 2003; Canene-Adams and others 2007; Bhattacharya and others 2010), which further supports efforts to develop functional foods containing whole fruits or vegetables rather than supplements containing purified phytochemicals.
Glucosinolates and ITCs can also have negative effects on health. When consumed in excess, glucosinolates can have goitrogenic effects. In 2009, for example, a woman was hospitalized with myxedema coma after reportedly eating 1 kg of raw bok choy every day for several months (Rabin 2010). Indeed, the goitrogenic properties of glucosinolates are well documented (McDanell and others 1988). The goitrogenic properties of canola prevented them from being used extensively as feed for livestock until varieties containing low levels of GLUs were developed (Olsen and Sørensen 1980). The cruciferous vegetables with the greatest goitrogenic activity are turnips and rutabagas; however, even these vegetables are believed to be only potentially harmful if consumed in excess, provided iodine intake is adequate (Steinmetz 2004).

In addition to the goitrogenic effects of glucosinolates, ITCs can be toxic at high levels (Mortazavi and others 2008). Fortunately, concentrations of ITC metabolites in blood plasma do not appear to increase with repeated intake of cruciferous vegetables beyond approximately three hours after ingestion, indicating that ITC metabolites are not accumulated in the body over time (Hanlon and others 2008; Hanlon and others 2009). These findings suggest that when consumed in moderation, the potential benefits to health of cruciferous vegetables make them a valuable tool in the pursuit of cancer prevention through the use of functional foods. Furthermore, the goitrogenic effects of consuming excessive quantities of cruciferous vegetables may be countered by supplementing the diet with iodine (Steinmetz 2004).

1.5 **Effects of processing cruciferous vegetables on health and sensory attributes**

Thermally processing cruciferous vegetables can have both positive and detrimental effects on their sensory properties as well as their ability to deliver ITCs
when consumed. Severe heat treatments, such as those used in the canning of tomato juice, degrade ITCs (Van Eylen and others 2007), producing odious volatile compounds. Not only does this reduce the cancer preventative potential of cruciferous vegetables, it also significantly hampers the sensory acceptability of products containing them. Sulfides produced when GLUs and ITCs are degraded have been attributed to the objectionable aromas in cooked cruciferous vegetables, especially those that are commonly cooked for long periods of time, such as cabbage or leafy greens (Hansen and others 1996).

On the other hand, mild heat treatments such as light steaming or blanching inactivate myrosinase, decreasing the pungency cruciferous vegetables, without significantly degrading GLUs and ITCs (Verkerk and others 2009). Since the pungency and bitterness of GLUs and ITCs are often undesirable (Engel and others 2002), these mild heat treatments could improve the sensory characteristics of cruciferous vegetables. In addition, relatively small amounts of sulfides in cruciferous vegetables actually contribute positively to perceived cooked broccoli flavor (Buttery and others 1976).

As with the sensory properties of cruciferous vegetables, mild heat treatments may actually improve their cancer preventative potential by increasing the delivery of ITCs. Many publications concerned with sulforaphane and broccoli assume that sulforaphane is the only hydrolysis product of glucoraphanin (Fahey, Zhang, and Talalay 1997; Shapiro and others 1998), but recent evidence suggests that when broccoli homogenates are made, glucoraphanin is not fully converted to sulforaphane (Mithen and others 2003; Matusheski, Juvik, and Jeffery 2004; Matusheski and others 2006). In addition to myrosinase, broccoli and broccoli sprouts have been shown to contain ESP activity (Petroski and Tookey 1982). This enzyme, in conjunction with myrosinase,
catalyzes the conversion of glucoraphanin into sulforaphane nitrile, rather than sulforaphane (Figure 1.3). Furthermore, unlike sulforaphane, sulforaphane nitrile has not been shown to have anticancer activity (Matusheski and Jeffery 2001). This means that the presence of ESP could inhibit the formation of sulforaphane, decreasing the cancer preventative potential of broccoli sprouts.

![Figure 1.3](image.png)

**Figure 1.3** Formation of sulforaphane nitrile by ESP.

Additional research has shown that the microbiota of the human gut has endogenous myrosinase activity (Getahun and Chung 1999). This means that if GLUs (or cooked cruciferous vegetables) are consumed, ITCs will be absorbed (Shapiro and others 1998; Vermeulen and others 2008). It is therefore reasonable to hypothesize that
blanching or lightly steaming broccoli sprouts to inactivate ESP (and myrosinase) may improve the bioavailability of sulforaphane by preventing the formation of sulforaphane nitrile.

To the contrary, however, subsequent research on the bioavailability of GLUs and ITCs has indicated that the bioavailability of GLUs is only about 10%, whereas that of raw broccoli is near 50% (Figure 1.4) (Conaway and others 2000; Shapiro and others 2001; Bheemreddy and Jeffery 2007; Hanlon and others 2008; Vermeulen and others 2008; Clarke and others 2011a, 2011b). It has also been demonstrated that consuming broccoli supplements lacking myrosinase concurrently with raw broccoli sprouts or broccoli powder containing active myrosinase dramatically improves the bioavailability of sulforaphane (Cramer and Jeffery 2011; Cramer, Teran-Garcia, and Jeffery 2011). This means that myrosinase (and possibly ESP as well) is active in broccoli during consumption and that functional foods designed for sulforaphane delivery should be formulated with myrosinase activity still intact.
Despite these findings, recent studies indicate that mild heat treatments may improve the bioavailability of sulforaphane from broccoli. Myrosinase has been shown to be somewhat more thermally stable than ESP, and heating broccoli and broccoli sprouts to temperatures between 60 and 70°C for 10 minutes has been shown to dramatically increase the amount of sulforaphane formed when broccoli is homogenized in water (Matusheski, Juvik, and Jeffery 2004). Furthermore, this effect was shown to be the result of inactivating ESP without damaging myrosinase. Thus, pretreating broccoli sprouts in this may be one strategy for improving the delivery of sulforaphane.

A second method for improving the bioavailability of sulforaphane beyond that in raw broccoli may be to utilize a foreign source of myrosinase that is not accompanied by ESP. Researchers studying ITCs and cruciferous vegetables have long utilized daikon radish sprouts as a naturally abundant source of myrosinase (Shikita and others 1999;
Matusheski and Jeffery 2001; Shapiro and others 2001). In addition, daikon radish sprouts do not contain measurable concentrations of ESP (Matusheski and others 2006). Therefore, steaming or blanching broccoli to inactivate enzymatic activity may result in increased sulforaphane delivery if the broccoli is combined with raw daikon radish sprouts, which contribute natural myrosinase.

In summary, whereas severe heat treatments have drastically negative effects on both the sensory properties of cruciferous vegetables and their ability to deliver ITCs, mild heat treatments may actually improve both of these properties. These heat treatments have the potential to reduce pungency in cruciferous vegetables and contribute desirable cooked flavor by limiting ITC formation and producing small amounts of sulfides, respectively. Furthermore, inactivating ESP by utilizing heat treatments may improve the bioavailability of sulforaphane in broccoli. Thus, the manner in which cruciferous vegetables are heat treated play an important role toward their sensory and healthful properties.

1.6 **Effects of carotenoids, isoflavones, and isothiocyanates on health**

Our novel soy-tomato-broccoli sprout beverage is based on a soy-tomato beverage previously developed with the group. The previously developed beverage contains high levels of lycopene and soy isoflavones, both of which have been shown to convey cancer preventative properties. By incorporating broccoli sprouts into the beverage, we hope to boost its anticancer effects.

Soy isoflavones have been extensively researched for their estrogenic and anticancer effects. Epidemiological evidence has suggested a relationship between soy intake and reduced rates of various cancers, including those of the breast and prostate.
Genistein, the most abundant aglycone isoflavone in soy, has been shown to inhibit the growth of various types of human and murine cancer cell lines *in vitro*, including leukemia, breast, colon, and prostate cancer cells (Messina, Persky, and Setchell 1994). Furthermore, animal studies have demonstrated the protective effect of soy isoflavones against various cancers, including those of the breast (Troll and others 1980; Barnes and others 1990; Hawrylewicz, Huang, and Blair 1991), liver (becker 1981, mokhtar 1988) prostate (Sharma and others 1992), stomach (Kim and others 1985), and bladder (Mokhtar and others 1988). The anticancer effects of genistein and other soy isoflavones is thought to stem largely from their inhibitory effect on tyrosine kinase, which tends to be overexpressed in cancer cells (Hunter and Cooper 1985), but isoflavones also interact with many targets downstream of growth factor receptors, such as phospholipase C-gamma, phosphatidylinositol kinase, and mitogen-activated protein kinase (Messina, Persky, and Setchell 1994).

Lycopene, the carotenoid responsible for the red color of tomatoes and many other fruits and vegetables, has also been shown to convey potent anticancer properties. Research on the cancer preventative effects of lycopene has mostly concentrated on prostate cancer, but evidence suggests that lycopene may prevent other types of cancer as well, including breast, cervical, ovarian, and liver cancers (A.V. Rao and L.G. Rao 2007). Epidemiological evidence has linked consumption of lycopene and tomatoes to reduced rates of prostate cancer (Giovannucci and others 2002), and *in vitro*, lycopene has been shown to inhibit the growth of various types of human prostate cancer cells (Kotake-Nara and others 2001). The primary mechanism by which lycopene inhibits growth of cancers appears to be its ability to trap reactive oxygen species, preventing damage to cellular
components, including DNA (A.V. Rao, Ray, and L.G. Rao 2006). Thermally processing tomatoes and combining lycopene with fat has been shown to increase lycopene bioavailability (Brown and others 2004; Unlu and others 2007), making our soy-tomato beverage with olive oil an excellent delivery system for lycopene.

A recent study has suggested that adding broccoli to tomato may increase the anticancer properties of tomatoes (Canene-Adams and others 2007). In the study, Male Copenhagen rats were fed diets containing lycopene, tomato, broccoli, and combinations of tomato and broccoli continuously for one month prior to subcutaneous prostate tumor implantation. Rats fed diets containing tomato powder had significantly lower prostate tumor weights after 18 weeks than those fed lycopene, even though the lycopene concentration in the two diets was the same. Furthermore, diets containing combinations of tomato and broccoli resulted in a further decrease in prostate tumor weight. These results have led us to hypothesize that adding broccoli sprouts to our soy-tomato beverage will further increase its cancer preventative potential.

**1.7 Research plan**

Thermally processing cruciferous vegetables has been shown to affect both their sensory properties and healthful benefits. Heat-treating broccoli sprouts to inactivate enzymatic activity slows the production of ITCs, which can provide a desirable sensory effect by reducing the intense pungency they convey. Conversely, inactivating myrosinase, which converts GLUs to ITCs, has been shown to decrease the bioavailability of ITCs and thus the cancer preventative potential of cruciferous vegetables. In addition, thermally processing cruciferous vegetables can cause degradation of sulfurous compounds, producing undesirable volatile compounds that
diminish sensory acceptability. Based on these considerations, our plan for developing a new soy-tomato-broccoli sprout beverage that represents the best compromise between cancer preventative potential and sensory acceptability consisted of three main parts:

1) Utilizing recent discoveries that mild heat treatments on cruciferous vegetables such as broccoli sprouts affect their cancer preventative potential, we developed four prototypes of freeze-dried broccoli sprout powder that vary in the way the sprouts are thermally processed before freeze-drying. The four prototypes developed were RP, SP, 60P, and SDP. We hypothesized that the different powders would vary in their pungency and overall aroma, which would affect their overall sensory acceptability.

2) In order to determine which of these broccoli sprout powder prototypes provided the best compromise between maximizing healthful potential and sensory acceptability, we conducted a sensory experiment in which we added the powders at a concentration of 1% in the soy-tomato juice and asked subjects to evaluate them for their sensory properties. We found that while the differences in sensory properties between the samples were subtle, processing methods clearly affected the sensory acceptability of the beverages.

3) Utilizing the data from the first sensory experiment, we chose to further develop the beverage containing SDP. To determine the maximum amount of broccoli sprout powder we could add to the soy-tomato juice before the sensory acceptability of the resulting beverage was significantly hampered, we conducted a second sensory experiment in which we varied the concentration of the broccoli sprout powder in the soy-tomato juice and asked subjects to evaluate the samples for their sensory properties.
Based on the results, we believe a concentration of approximately 0.5% will provide the best compromise between GLU/ITC delivery and sensory acceptability.

The following chapters each describe these three main endeavors in greater detail. In summary, this project demonstrates how food processing technology, food chemistry, and sensory science can be used to further the goal of developing bioactive-enhanced functional foods that provide both healthful effects and pleasing sensory properties.
CHAPTER 2: MAXIMIZING SULFORAPHANE DELIVERY AND SENSORY ACCEPTABILITY OF A NOVEL SOY-TOMATO-BROCCOLI SPROUT BEVERAGE

2.1 Abstract

Recent research has elucidated the potent anticancer effects of sulforaphane, an isothiocyanate (ITC) especially abundant in broccoli sprouts. These findings have encouraged us to develop a novel soy-tomato-broccoli sprout beverage designed for cancer prevention based on an existing soy-tomato beverage previously developed by our group. To avoid the ITC degrading effects of processing broccoli sprouts using thermally intensive methods, we chose to develop freeze-dried broccoli sprout powders that can be mixed with our soy-tomato juice to boost its cancer preventative potential.

Our goal was to develop a beverage that represents the best compromise between maximizing sulforaphane delivery and retaining sensory acceptability. To do this, we first confirmed recent research that indicates mild heat treatments boost the formation of sulforaphane in broccoli in vitro. Based on our findings, we then designed four prototypes of our broccoli sprout powder that differ in the manner in which the broccoli sprouts are heat treated before freeze-drying: 1) raw broccoli sprout powder (RP), 2) steamed broccoli sprout powder (SP), 3) 60ºC pretreated broccoli sprout powder (60P), and 4) 90% steamed broccoli sprout powder plus 10% raw daikon sprout powder (SDP).
To determine how the powders vary in the extent to which they deliver ITCs \textit{in vitro}, we measured the formation of sulforaphane in our soy-tomato juice after the addition of the broccoli sprout powders. We found that the 60P contributed the most sulforaphane, followed by RP, SDP and SP, respectively. Interestingly, the SDP showed a delayed formation of sulforaphane into the beverage as compared to the 60P and RP, which we hypothesized may have a positive effect on sensory properties by limiting the formation of pungent ITCs until after the beverage has been consumed.

Since the manner in which cruciferous vegetables are cooked also affects their sensory properties, we hypothesized that our various broccoli sprout powders would produce beverages that vary in their overall sensory acceptability. Next, we performed two sensory experiments to help us choose the freeze-dried powder that produces the soy-tomato-broccoli sprout beverage that represents the best compromise between cancer preventative potential and sensory acceptability.

In the first experimental phase, we added the powders to previously canned soy-tomato juice at a level of 1% by weight and asked subjects to evaluate the overall acceptability of the beverages. We found that in all cases the broccoli sprout powder significantly decreased the sensory acceptability of the soy-tomato juice. We also saw that although differences between the samples were subtle, the SDP beverage showed nearly identical results to the SP beverage despite containing active myrosinase. In our second experimental phase, we chose to add the SDP to newly canned soy-tomato juice at varying concentrations. We found that a concentration of approximately .5% broccoli sprout powder in the juice provides a sensory acceptability that was not significantly different from the control of juice alone. Based on these results, we believe that adding
the SDP to the juice at a concentration of approximately .5% produces the best compromise between sensory acceptability and healthful potential of the beverage.

2.2 Introduction

Functional foods are designed to provide healthful benefits that extend beyond their nutritional properties. Recently, a great deal of interest has been placed into the development of functional foods containing elevated levels of phytochemicals believed to have anticancer effects. One such phytochemical is sulforaphane, an ITC especially abundant in broccoli and broccoli sprouts (Fahey, Zhang, and Talalay 1997; Tian, Rosselot, and Schwartz 2005). Sulforaphane has been shown to be a potent inducer of phase-II detoxification enzymes, slowing the progression of cancerous cells in numerous in vitro and in vivo studies (Conaway, Yang, and Chung 2002). Furthermore, the results of numerous studies have indicated that consumption of whole fruits or vegetables containing elevated levels of bioactive phytochemicals may have a more potent anticancer effect than consumption of purified phytochemicals (Keck, Qiao, and Jeffery 2003; Canene-Adams and others 2007; Bhattacharya and others 2010). Considering these findings, it is clear why there is such great interest in developing functional foods formulated with broccoli containing elevated levels of sulforaphane.

Previously, our group developed a soy-tomato beverage formulated with elevated levels of lycopene and soy isoflavones, both of which have been shown to convey cancer preventative properties (Messina, Persky, and Setchell 1994; Canene-Adams and others 2007). A recent study indicating that there may be an additional cancer preventative effect when tomato and broccoli are consumed together (Canene-Adams and others 2007) has led us to hypothesize that the addition of broccoli sprouts into our soy-tomato
beverage may dramatically boost its cancer preventative potential since broccoli sprouts contain especially high levels of sulforaphane (Fahey, Zhang, and Talalay 1997; Tian, Rosselot, and Schwartz 2005).

The value of a functional food should be measured not only by its ability to deliver the phytochemical or prevent the disease in question, but also by the acceptability of its sensory properties. Therefore, our goal was to formulate our new soy-tomato-broccoli sprout beverage to represent the best compromise between maximizing benefits to health and sensory acceptability. The next chapter focuses primarily on the sensory properties of the beverage; first, this chapter deals with how we developed several prototypes of our functional beverage based on attempting to maximize the beverage’s delivery of sulforaphane. To do this, we first tested and implemented recent discoveries that have shown the manner in which broccoli and broccoli sprouts are processed may dramatically affect the bioavailability of sulforaphane.

2.2.1 Maximizing sulforaphane delivery via freeze-dried broccoli sprout powders

ITCs exist in raw cruciferous vegetables as their glycosylated precursors, glucosinolates (GLUs). When the cells of cruciferous vegetables are breached, GLUs mix with the enzyme myrosinase, which converts them to ITCs. Figure 2.1 shows the action of myrosinase on glucoraphanin, the GLU precursor to sulforaphane. Myrosinase is a β-thioglucosidase that converts glucoraphanin into an unstable intermediate, which then undergoes an uncatalyzed Lossen-type rearrangement to form sulforaphane (Fenwick and others 1982). There are therefore several considerations that must be made in order to maximize the delivery of sulforaphane in a functional beverage containing broccoli. First, broccoli containing high concentrations of glucoraphanin must be chosen. Secondly, the
broccoli must be processed in a way that minimizes the degradation of GLUs and ITCs. Finally, the beverage must be formulated in a way that maximizes the bioavailability of sulforaphane once the beverage is consumed.

Figure 2.1 Formation of sulforaphane nitrile by ESP.

To address the first consideration, we chose to use broccoli sprouts, which contain much greater concentrations of glucoraphanin than mature broccoli (Fahey, Zhang, and Talalay 1997; Tian, Rosselot, and Schwartz 2005). This is especially true of BroccoSprouts, a cultivar of broccoli sprouts developed by researchers at Johns Hopkins University. BroccoSprouts are advertised to contain high levels of “SGS” (Sulforaphane
Glucosinolate, another term for glucoraphanin) and can be purchased at many local supermarkets.

Secondly, to maximize the delivery of the sulforaphane from the broccoli sprouts they must be processed in a manner than does not degrade GLUs or ITCs. Unfortunately, recent research has shown that GLUs and ITCs are relatively thermolabile (Van Eylen and others 2007). The degree of thermal processing normally used in the canning of tomato juice, for example, would degrade GLUs and ITCs, so they cannot simply be added to our soy-tomato juice before canning. Not only would the process diminish the cancer preventative potential of the broccoli sprouts, but also it would result in the formation of odious volatile compounds that would significantly lower the sensory acceptability of the beverage. To avoid this issue we chose to develop a freeze-dried powder of broccoli sprouts that can be made using methods that are not thermally intensive. This powder can be added to our soy-tomato juice just before consumption to create a soy-tomato-broccoli sprout beverage that provides high levels of soy isoflavones, lycopene, and sulforaphane.

Beyond simply preventing the degradation of GLUs and ITCs, our goal was to formulate a soy-tomato-broccoli sprout beverage in a way that maximizes the delivery of sulforaphane, and recent research has indicated that mild heat treatments may actually improve the formation of sulforaphane from broccoli sprouts, possibly increasing their bioavailability upon consumption.

Many publications concerned with sulforaphane and broccoli assume that sulforaphane is the only hydrolysis product of glucoraphanin (Fahey, Zhang, and Talalay 1997; Shapiro, Fahey, Wade, Stephenson, and Talalay 1998), but recent research suggests
that this may be a faulty assumption. When broccoli or broccoli sprouts are homogenized in water and the homogenate is incubated to allow enzymatic activity, the amount of sulforaphane formed is actually relatively low compared to the amount of glucoraphanin present in the raw broccoli. This indicates that the glucoraphanin is not being fully converted to sulforaphane (Mithen and others 2003; Matusheski and others 2006). In addition to myrosinase, broccoli and broccoli sprouts have been shown to contain an enzyme called the epithiospecifier protein (ESP) (Petroski and Tookey 1982). This enzyme catalyzes the conversion of the unstable intermediate compound mentioned earlier into sulforaphane nitrile, rather than sulforaphane (Figure 2.1). Furthermore, unlike sulforaphane, sulforaphane nitrile has not been shown to have anticancer activity (Matusheski and Jeffery 2001). This means that the presence of ESP could inhibit the formation of sulforaphane, decreasing the cancer preventative potential of broccoli sprouts.

Additional research has shown that the microbiota of the human gut has endogenous myrosinase activity (Getahun and Chung 1999). This means that if GLUs (or cooked cruciferous vegetables) are consumed, ITCs will be absorbed (Shapiro, Fahey, Wade, Stephenson, and Talalay 1998; Vermeulen and others 2008). From this evidence, it is therefore reasonable to hypothesize that blanching or lightly steaming broccoli sprouts to inactivate ESP (and myrosinase) may improve the bioavailability of sulforaphane by preventing the formation of sulforaphane nitrile.

To the contrary, however, subsequent research on the bioavailability of GLUs and ITCs has indicated that the bioavailability of GLUs is only about 10%, whereas that of raw broccoli is near 50% (Figure 2.2) (Conaway and others 2000; Shapiro and others
Studies performed in conjunction with our laboratory have shown that consumption of raw broccoli sprouts results in absorption of approximately four times more sulforaphane and erucin in urine than consumption of broccoli supplements lacking myrosinase activity (Clarke and others 2011a). A subsequent study showed that consumption of the raw broccoli sprouts sped the absorption of sulforaphane and resulted in decreased histone deacetylase activity as compared to consumption of the broccoli supplements, even though the dose of GLUs was the same (Clarke and others 2011b). This means that myrosinase is active in broccoli sprouts during consumption; therefore, functional foods designed for sulforaphane delivery should be formulated with myrosinase activity still intact.
Figure 2.2  Bioavailability of isothiocyanates and glucosinolates in cooked and raw broccoli. Adapted from Shapiro and others (2001).

With these discoveries in mind, there may still be ways to improve the delivery of sulforaphane from broccoli beyond simply consuming it raw. Figure 2.2 indicates that consumption of broccoli in which GLUs have been fully converted to ITCs may maximize the delivery of ITCs. Accordingly, at least one functional beverage has been developed in which a sulforaphane-rich preparation was added (Egner and others 2011). We chose not to create a sulforaphane-rich powder because like other ITCs, sulforaphane contributes intense pungency to foods in high concentrations, which is often undesirable (Engel and others 2002). In fact, the study mentioned above reported that some subjects consuming their sulforaphane-rich beverage experienced nausea and/or vomiting due to its sensory properties (Egner and others 2011).

Fortunately, there are still at least two viable strategies for improving the bioavailability of sulforaphane from broccoli beyond simply consuming it raw that do not
have such drastically negative sensory effects. First, myrosinase has been shown to be somewhat more thermally stable than ESP, and heating broccoli and broccoli sprouts to temperatures between 60 and 70°C for 10 minutes has been shown to dramatically increase the amount of sulforaphane formed when broccoli is homogenized in water (Matusheski, Juvik, and Jeffery 2004). Furthermore, this effect was shown to be the result of inactivating ESP without damaging myrosinase (Matusheski, Juvik, and Jeffery 2004). Thus, pretreating broccoli sprouts in this manner before freeze-drying may be one strategy for improving the delivery of sulforaphane in our soy-tomato-broccoli sprout beverage.

A final method for improving the bioavailability of sulforaphane beyond that in raw broccoli may be to utilize a foreign source of myrosinase that is not accompanied by ESP. Researchers studying ITCs and cruciferous vegetables have long utilized daikon radish sprouts as a naturally abundant source of myrosinase (Shikita and others 1999; Matusheski and others 2001; Shapiro and others 2001). In addition, daikon radish sprouts do not contain measurable concentrations of ESP (Matusheski and others 2006). We therefore hypothesized that a way to improve the delivery of sulforaphane in our beverage may be to lightly steam the broccoli sprouts to inactivate ESP (and all enzymatic activity) and add them to a small percentage of raw daikon radish sprouts before freeze-drying, ensuring that myrosinase activity, but not ESP, will remain in the powder.

With these thoughts in mind, we first verified the effects of heating broccoli sprouts to 60°C for 10 minutes on the formation of sulforaphane *in vitro*. Secondly, we confirmed that daikon radish sprouts are as effective as purified myrosinase for
converting glucoraphanin to sulforaphane in homogenates of steamed broccoli sprouts. Finally, we developed four prototypes of freeze-dried broccoli sprout powder that can be added to our previously developed soy-tomato juice to create four new soy-tomato-broccoli sprout beverages: 1) raw broccoli sprout powder (RP), 2) steamed broccoli sprout powder (SP), 3) 60ºC pretreated broccoli sprout powder (60P), and 4) 90% steamed broccoli sprout powder plus 10% raw daikon sprout powder (SDP). Since these powders vary in the extent to which myrosinase and ESP are active, we hypothesized that they would vary in the extent to which sulforaphane is produced when they are mixed with our soy-tomato juice. We also hypothesized that this difference in sulforaphane formation would have an effect on both the healthful properties and sensory characteristics of the resulting beverage. To demonstrate these differences, we added our freeze-dried broccoli sprout powders to our soy-tomato juice and measured the formation of sulforaphane over time. The results of these experiments are discussed in the following section.

2.2.2 Determining sensory acceptability of soy-tomato-broccoli sprout beverages

The development of functional foods provides a unique challenge when compared to that of supplements or drugs in that the sensory characteristics of the food must be considered in addition to its effects on health. GLUs and ITCs can contribute both positive and negative effects to the sensory properties of foods containing cruciferous vegetables. In some cases, the pungent aroma of ITCs is a desired component of the taste and flavor of a food. Mustard, the world’s second most traded spice in terms of market value, as well as horseradish and wasabi owe their characteristic pungency to allyl ITC (Weiss 2002). The bitterness of GLUs can also be important for the characteristic flavor
of certain foods. Arugula, for example, has a unique bitter flavor that distinguishes it from other salad greens, which stems primarily from its relatively high content of GLUs such as glucoerucin (Pasini and others 2011).

In other cases, the pungent aroma of ITCs and bitterness of GLUs can contribute negatively to sensory acceptance. Pungency and bitterness from GLUs and ITCs has been shown to negatively impact the sensory qualities of cauliflower and other cruciferous vegetables (Engel and others 2002). Many cruciferous crops have been bred to produce lower levels of GLUs for this reason (Verkerk and others 2009). Conversely, broccoli and broccoli sprouts have been bred for increased GLU content due to their healthful effects. The utilization of broccoli in a functional food therefore provides an additional challenge.

Based on research indicating that allowing myrosinase to fully convert GLUs to ITCs may maximize ITC bioavailability (Shapiro and others 2001), at least one functional beverage consisting of broccoli sprout-derived sulforaphane has been developed (Egner and others 2011). Unfortunately, fully converting GLUs to ITCs also produces a product that is extremely pungent and may cause nausea and vomiting upon consumption (Egner and others 2011).

Previously we described how intense heat treatments can degrade ITCs in cruciferous vegetables, reducing their cancer preventative potential, whereas mild heat treatments may actually improve the bioavailability of ITCs. Likewise, heat treatments have a similar effect on the sensory properties of cruciferous vegetables. Severe heat treatments, such as those used in the canning of tomato juice, degrade ITCs (Van Eylen and others 2007), producing odious volatile compounds. Not only does this reduce the cancer preventative potential of cruciferous vegetables, it also significantly hampers the
sensory acceptability of products containing them. Sulfides produced when GLUs and ITCs are degraded have been attributed to the objectionable aromas in cooked cruciferous vegetables, especially those that are commonly cooked for long periods of time, such as cabbage or leafy greens (Hansen and others 1996).

On the other hand, mild heat treatments such as light steaming or blanching inactivate myrosinase, decreasing the pungency cruciferous vegetables, without significantly degrading GLUs and ITCs (Verkerk and others 2009). In fact, whereas steamed broccoli sprouts have a flavor very similar to broccoli, raw broccoli sprouts have a flavor more reminiscent of radishes due to the intense pungency that myrosinase produces upon chewing. Since pungency and bitterness are often undesirable, these mild heat treatments could improve the sensory characteristics of cruciferous vegetables. In addition, relatively small amounts of sulfides in cruciferous vegetables actually contribute positively to perceived cooked broccoli flavor (Buttery and others 1976).

Based on these findings, it was reasonable for us to hypothesize that our freeze-dried broccoli sprout powders, which differed in the manner in which the sprouts were heat treated before freeze-drying, would have varying sensory properties. We next performed two sensory experiments in order to test this hypothesis and to determine which preparation produced the best combination of hypothesized cancer preventative potential and sensory acceptability.

In the first experimental phase, we tested four freeze-dried broccoli sprout powders, RP, 60P, SP, and SDP, in a previously canned soy-tomato juice at a concentration of 1% by weight and asked subjects to evaluate the beverages for overall acceptability, amount of broccoli flavor, and amount of spiciness/pungency. We then
asked subjects to rank the samples in order of their overall preference. Based on the results of this experiment, we chose to further develop the SDP beverage.

In the second experimental phase, we added the SDP to a newly canned soy-tomato juice at concentrations of 0, .25, .5, and 1% and asked subjects to evaluate the samples in the same way as in the first experiment. Based on our findings, we feel that adding the SDP to the soy-tomato juice at a level of approximately .5% represents the best compromise between maximizing ITC delivery and retaining sensory acceptability.

2.3 Materials and methods

2.3.1 Effects of 60°C pretreatments on sulforaphane formation in broccoli sprouts

BroccoSprouts brand broccoli sprouts were purchased at a local Giant Eagle supermarket. For the 60°C pretreatment, the broccoli sprouts were placed in a plastic zip-top bag, from which the air was removed before sealing. The bag of sprouts was heated in a pot of water held at 60°C for 10 minutes, after which the bag of sprouts was placed in an ice bath.

Raw and pretreated sprouts were then used to make broccoli sprout homogenates in water. Approximately 20 g raw and pretreated sprouts were blended with 300 ml deionized water in a Waring commercial laboratory blender for five minutes. Samples of the homogenates were aliquoted into glass vials and incubated in a water bath at 45°C for two hours to allow for enzymatic conversion. After incubation, ITCs in the homogenates were quantified as described below.

2.3.2 Utilization of raw daikon radish sprouts as a source of myrosinase

BroccoSprouts brand broccoli sprouts were purchased at a local Giant Eagle supermarket, and Kaiware brand daikon radish sprouts were purchased at a local
Japanese market. The broccoli sprouts were steamed for five minutes in a simple steaming apparatus consisting of a pot of boiling water on a hot plate, a metal colander, and a metal bowl on top to serve as a lid to trap steam. After steaming, the sprouts were cooled by briefly rinsing with cold tap water. Homogenates of steamed broccoli sprouts were made by blending approximately 20 g steamed sprouts with approximately 150 ml deionized water. Approximately 3 g of raw daikon radish sprouts were added to half the mixtures before homogenizing. The mixtures without daikon radish sprouts were treated with approximately 10 mg purified myrosinase from white mustard seed, which was purchased from Sigma-Aldrich. Aliquots of the homogenates were made in glass vials, and the samples were incubated in a water bath at 45°C for two hours. After incubation, ITCs in the samples were quantified as described below.

2.3.3 Extraction and quantification ITCs in broccoli sprout homogenates

To determine the concentrations of ITCs formed in the broccoli sprout homogenates, approximately four grams of homogenate was extracted twice with 10 ml dichloromethane. After the addition of dichloromethane, the samples were further homogenized using a probe sonicator. The samples were then centrifuged at 1200*g for 10 minutes, and the solvent was removed. Pooled solvent was dried under nitrogen. For quantification of ITCs, the 2-mercaptoethanol conjugation method described by Vermeulen, and others was used. Extracts were reconstituted in 1 ml dichloromethane plus .5 ml “Vermeulen reagent,” consisting of 20 mM triethylamine and 200 mM 2-mercaptoethanol in dichloromethane. The samples were incubated in a water bath at 30°C for one hour, after which the solvent was removed under nitrogen. Samples were
reconstituted in 1 ml methanol and filtered through .2 µm syringe filters into high performance liquid chromatography (HPLC) vials.

For HPLC analysis, samples were injected onto a Waters Symmetry C18 column (3.5 µm pore size, 4.6 x 75 mm) using a Waters 2695 separations module and detected using a Waters 996 photo diode array detector. Samples were separated using a linear gradient from 100% water containing .1% formic acid to 50% acetonitrile containing .1% formic acid over 15 minutes. ITC-mercaptoethanol conjugates were quantified at 271 nm using a standard curve (Appendix E).

Sulforaphane, erucin, and iberin standards were purchased from LKT laboratories. 2-mercaptoethanol and formic acid were purchased from Sigma-Aldrich, and dichloromethane, acetonitrile, and HPLC-grade water were purchased from Fisher Scientific. Triethylamine was purchased from JT Baker.

2.3.4 Spike-recovery tests for reproducibility of quantifying ITCs by 2-mercaptoethanol conjugation

The reproducibility of the 2-mercaptoethanol conjugation method for quantifying ITCs was measured by performing spike-recovery tests on the broccoli homogenates. Standards of sulforaphane, iberin, erucin, allyl ITC, and phenethyl ITC were made in water, and known quantities were added to the broccoli sprout homogenates. Allyl and phenethyl ITC standards were purchased from Sigma-Aldrich, and sulforaphane, erucin, and iberin standards were purchased from LKT Laboratories. Spiked and unspiked samples were extracted and analyzed by HPLC using the 2-mercaptoethanol conjugation method as described above. ITC-mercaptoethanol conjugates were quantified at 271nm. Differences in peak area between the spiked and unspiked samples were compared to the
peak area expected from standard curves of each of the ITC standards (Appendix E). The recovery rate was calculated as the difference in peak area between the spiked and unspiked samples divided by the peak area difference expected.

2.3.5 Production of freeze-dried broccoli sprout powders

2.3.5.1 Heat treatments

BroccoSprouts brand broccoli sprouts were purchased at a local Giant Eagle supermarket. The sprouts were washed in a solution of 200 ppm sodium hypochlorite and then rinsed thoroughly with cold tap water. The sprouts were then steamed by placing them in a metal colander and placing the colander over a pot of boiling water on a hot plate. A metal bowl or lid was placed on top of the colander to trap steam. Sprouts were steamed for five minutes over a rolling boil. The colander was then taken off the steam, and excess moisture was removed from the sprouts by tapping the colander over paper towels on the workbench. The sprouts were then flash frozen and freeze-dried, as described below.

To conduct the 60°C pretreatment, a pot was filled with hot tap water, and the water was heated to just over 60°C on a hot plate. A digital thermometer was used to ensure that the heat output of the hot plate kept the water at exactly 60°C throughout the pretreatment. After being washed with a solution of 200 ppm sodium hypochlorite and rinsed with cold tap water, the broccoli sprouts, in batches of up to 4 oz. each, were placed in the water for 10 minutes. After the pretreatment, the sprouts were dried by pouring them into a colander and tapping the colander over paper towels on the workbench to remove excess moisture. The sprouts were then flash frozen and freeze-dried, as described below.
2.3.5.2 Flash freezing and freeze-drying

Kaiware brand daikon radish sprouts were purchased at a local Japanese market. Raw broccoli sprouts and daikon sprouts were washed in a solution of 200 ppm sodium hypochlorite and rinsed with cold tap water. Raw, steamed, and 60°C pretreated broccoli sprouts and raw daikon sprouts were each flash frozen with liquid nitrogen before freeze drying.

Freezing the sprouts in large clumps can greatly increase the amount of time required to remove all moisture during freeze drying; to prevent this issue, the sprouts were spread out in a metal pan before being flash frozen with liquid nitrogen. This forms a thin sheet of frozen broccoli sprouts, which can then be broken with a metal spatula and transferred to glass lyophilizer flasks. When performing this step, one should place a pot holder or oven mitt under the metal pan, or else the bench top will become extremely cold, causing water to condense and freeze on the bench top. Once the frozen sprouts were placed into lyophilizer flasks, they were freeze-dried using a Labconco Freeze Dry System/Lyph Lock 4.5 lyophilizer for up to 48 hours, until the sprouts were completely dry.

2.3.5.3 Grinding freeze-dried sprouts into powders

A large mortar and pestle were used to grind the freeze-dried sprouts into fine powders in batches of a few grams each. When grinding the freeze-dried sprouts, one should first lightly crush the sprouts with the pestle until the sprouts can easily be moved around the mortar. Then, one should use the pestle to apply light pressure to the sprouts in a circular motion around the mortar. The dried sprouts are very brittle and will form a fairly fine powder if they are simply moved around the mortar in this way. Once a fairly
fine, homogenous powder is made, heavy pressure should be applied with the pestle as it is twisted against the mortar to grind the sprouts into a very fine powder.

After the dried sprouts were ground into fine powders, they were packaged and stored in the refrigerator. The steamed sprout powder was added to the raw daikon radish powder at a ratio of 90:10 to form the SDP.

2.3.6 Measuring formation of sulforaphane in soy-tomato juice after the addition of broccoli sprout powders

Previously canned soy-tomato juice and broccoli sprout powders were weighed so that the resulting mixture would contain .5% broccoli sprout powder by weight. The broccoli sprout powders were mixed thoroughly into the soy-tomato juice, and a timer was immediately started. The homogenized soy-tomato-broccoli sprout mixtures were then aliquoted into premeasured glass centrifuge tubes using a 5-ml pipette to transfer approximately 2ml of the mixture per aliquot. The tubes were then weighed so that the weight of each aliquot could be determined. After serial times, the tubes were placed into boiling water for three minutes to inactivate myrosinase, after which the tubes were placed into an ice water bath.

To quantify the sulforaphane formed in each aliquot, the samples were extracted twice with 5 ml of dichloromethane. After each addition, the samples were homogenized using a probe sonicator. The aliquots were then centrifuged at (centrifuge speed) for 10 minutes before the dichloromethane fractions were removed using glass Pasteur pipettes. From the pooled extracts, one ml of each was added to .5 ml “Vermeulen reagent,” consisting of 20 mM triethylamine and 200 mM 2-mercaptoethanol in dichloromethane. The samples were then allowed to incubate at room temperature for one hour before the
dichloromethane was evaporated under nitrogen. During this step, samples were placed in a block heater over very low heat to counter the effects of evaporative cooling. Dried samples were stored until time for analysis.

For analysis of sulforaphane concentration by HPLC-photo diode array (PDA), dried samples were reconstituted in 1 ml methanol, which solubilizes ITCs but causes carotenoids to precipitate. Samples were then filtered through .2 µm syringe filters before injection on the HPLC. Samples were separated by HPLC using the water-acetonitrile gradient described above.

2.3.7 Analysis of GLUs in broccoli sprout powders

Approximately 100 mg samples of freeze-dried powders were first extracted with 10 ml boiling deionized water. The samples were held for three minutes before being placed in an ice water bath. The samples were vortexed, sonicated in a water bath, and centrifuged at 1200*g for 10 minutes. The aqueous fraction was collected, and the samples were extracted in this way twice more with 10 ml room temperature deionized water. The pooled fractions were diluted 100 times before being filtered through .2 µm nylon syringe filters into HPLC vials. The samples were analyzed by liquid chromatography-mass spectrometry (LCMS) as described below.

Samples were separated on a Zorbax SB-CN stable bond cyanopropyl column (5 µm pore size, 4.6 x 250 mm) using a gradient in which solvent A was .1% aqueous formic acid and sample B was acetonitrile with .1% formic acid. The flow rate was 1.5 ml/min. The gradient began at 100% A for three minutes, after which the gradient was increased to 10% B for one minute and then 50% B over four minutes. The column was
flushed by increasing the gradient to 95% B over one minute, and then the column was equilibrated to 100% A for three minutes.

MS analysis was performed on a QTrap 5500 from AB Sciex, Concord, Canada. The nebulizing gas was zero grade air at 60 psi. The desolvation gas was nitrogen at 55 psi. The curtain gas was also nitrogen at 30 psi. The temperature was set to 600°C, and the collision activated dissociation setting was set to medium. The Ion spray potential was 4.5 kV. The entrance potential was 10 V. The collision energy was 27 eV, and the collision cell exit potential was 11 V. Transition masses were 436>97 for glucoraphanin, 422>97 for glucoiberin, and 420>97 for glucoerucin.

2.3.8 Canning of soy-tomato juice

New soy-tomato juice was canned in the Ohio State Food Industries Center Pilot Plant. The juice consisted of 98.89% moderately-high lycopene tomato juice, .3% salt, 1% Buitoni extra light tasting olive oil, and .11% Solbar 40S soy isoflavone concentrate. The tomato juice used was previously canned by our group. The olive oil and salt were purchased at a local Giant Eagle supermarket, and the soy isoflavone concentrate was provided by Solbar Plant Extracts.

The juice was produced by first mixing the soy isoflavone concentrate, salt, and olive oil into a small portion (approximately one half gallon) of the tomato juice using an immersion blender. This step helps to prevent clumping of the soy isoflavone concentrate. Next, this portion was mixed into the rest of the tomato juice. In total, four gallons of juice were made. The juice was fed through a hopper and a positive displacement pump into a colloidal mill, which served to homogenize the olive oil into the tomato juice. The soy-tomato juice was collected as it exited the colloidal mill and
was next transferred to a steam-jacketed kettle. The juice was heated to 95°C before being poured into #300 cans. The cans were then fed through an automatic can sealer and then placed in a vertical still retort. The cans were retorted at 94°C for 15 minutes before being cooled.

2.3.9 Analysis of lycopene in soy-tomato juices

Lycopene concentration in the new soy-tomato juice was quantified by extraction and analysis by HPLC-PDA. Aliquots of approximately 1.5 g juice were transferred to 11 ml glass centrifuge tubes, and 5 ml methanol was added to each. The samples were mixed with a probe sonicator and then centrifuged at 1200*g for 10 minutes. The methanol fractions were removed, and the samples were then extracted three times with 5 ml 1:1 hexane:acetone. Each time, the samples were mixed with a probe sonicator and centrifuged at 1200*g for 10 minutes before the organic fractions were removed. The hexane layers from the pooled extracts were brought up to 10 ml using volumetric flasks, and from this 1 ml of hexane extract was dried under nitrogen. The samples were brought up in 1 ml 1:1 methyl tert-butyl ether (MTBE):methanol by adding the MTBE first. The samples were then filtered through .2 µm nylon syringe filters into HPLC vials.

Samples were injected onto a Waters YMC Carotenoid S-5 C30 HPLC column (4.6x150 mm) using a Waters 2695 separations module and detected using a Waters 996 photo diode array detector. The samples were separated using the following gradient conditions: Solvent A consisted of 88% methanol, 5% MTBE, 5% water, and 2% of a 2% ammonium acetate solution. Solvent B consisted of 78% MTBE, 20% methanol, and 2% of a 2% ammonium acetate solution. The gradient started at 10% solvent B and increased to 100% B over 11 minutes. The gradient was held at 100% B for 1.5 minutes, and then
the gradient was lowered to 10% B over five more minutes. The flow rate was 1.7 ml/min. Lycopene was quantified at 471 nm using a standard curve (Appendix E). All solvents were purchased from Sigma-Aldrich. Lycopene standard was purified from tomato paste by other members of the group.

2.3.10 Analysis of isoflavones in soy-tomato juices and soy isoflavone concentrate

The concentrations of soy isoflavones in the soy isoflavone concentrate used to make the newly canned soy-tomato juice were quantified by extraction and analysis by HPLC-PDA. Approximately 30 mg soy isoflavone concentrate was extracted three times with 2.5 ml 60% acetonitrile. Each time, samples were sonicated in a cold water bath for 20 minutes and centrifuged at 1200*g for 10 minutes. Pooled extracts were brought up to 25 ml with acetonitrile, and 100 µl of this collection was dried under nitrogen. The dried samples were brought up in 1 ml methanol, filtered through .2 µm nylon syringe filters into HPLC vials, and analyzed by HPLC as described below.

Newly canned soy-tomato juice was extracted in a similar manner as the soy isoflavone concentrate. Approximately 2.5 g juice was extracted three times with 7 ml acetonitrile. Each time, the samples were sonicated with a probe sonicator and centrifuged at 1200*g for 10 minutes. Pooled extracts were brought up to 25 ml acetonitrile using volumetric flasks, and 1ml of this extract was dried under nitrogen. Samples were reconstituted in 1 ml methanol, filtered through .2 µm nylon syringe filters into HPLC vials, and analyzed by HPLC as described below.

Samples to be analyzed for isoflavone concentrations were injected onto a Waters Symmetry C18 column (3.5 µm pore size, 4.6 x 75 mm) using a Waters 2695 separations module and detected using a Waters 996 photo diode array detector. The samples were
separated using the following gradient. Solvent A was 1% acetic acid in water, and solvent B was .1% formic acid in acetonitrile. The flow rate was 1.0 ml/min. The gradient began at 10% B for one minute and was increased to 35% B over 22 minutes. The gradient was further increased to 75% B over three more minutes. Then, the gradient was reduced to 10% B over three minutes and held there for 6 minutes. Daidzin, glycitin, genistin, daidzein, and genistein standards were made in methanol and used to create standard curves (Appendix E). Daidzin and daidzein were quantified at 249 nm. Glycitin and genistin were quantified at 259 nm, and genistein was quantified at 263 nm. Solvents were purchased from Sigma-Aldrich, and isoflavone standards were purchased from LC Laboratories.

2.3.11 Sensory studies

2.3.11.1 Approval for institutional review board (IRB) exemption

Both sensory studies were approved for exemption from IRB review under category six. The project number for the sensory studies was 2001E0609. Determination letters for IRB exemption are attached (Appendix C).

2.3.11.2 Recruitment

Subjects were eighteen years or older and were recruited primarily through email. Subjects were offered a $5 Target gift card for participating. Additional subjects were recruited from the lobby of the Parker food science building to replace recruited subjects who were not present. A total of 80 subjects were recruited for both sensory studies, of which the tests were administered to 72 and 80 subjects in the two sensory tests, respectively. Copies of the recruitment letters used are attached (Appendix D).
2.3.11.3 Administration of sensory experiments

Sensory studies were administered in private sensory booths at the Ohio State Department of Food Science sensory testing facility. Subjects were given a brief orientation of sensory terms and structure of the test and were asked to sign a consent form (Appendix B). Sensory studies were conducted in rounds of 10 subjects each, every half hour. Subjects were given samples of soy-tomato-broccoli sprout beverages and were asked to evaluate each sample on overall acceptability on a nine-point hedonic scale. Subjects were then asked to evaluate the amount of broccoli flavor and amount of pungency/spiciness of the beverages on a five-point Just About Right (JAR) scale. Finally, subjects were asked to rank the samples in order of their overall preference and provide their age and gender (Appendix A). Subjects were then given a $5 Target gift card for participating.

Samples were administered to subjects by adding preweighed aliquots of broccoli sprout powders to the soy-tomato juice in order to make enough of each sample for 10 subjects at a time. The mixed soy-tomato-broccoli sprout beverages were then dispensed into sample cups labeled with randomly generated numeric codes. The samples were presented to subjects in random order, and the samples were presented under a red light to help subjects evaluate the samples on flavor rather than appearance.

2.4 Results and discussion

2.4.1 Analysis of ITCs by conjugation to 2-mercaptoethanol and detection with HPLC-PDA

In order to adequately determine the effects of the processing methods described on sulforaphane formation in broccoli sprouts, it was necessary to have accurate and
reliable methods for quantifying sulforaphane concentration in food samples. As discussed previously, there are many methods available for analyzing ITCs, but the simplest and most reliable methods involve the use of HPLC coupled with mass spectrometry (LCMS) or PDA. Since LCMS is not always readily available, we chose to use HPLC coupled with PDA to quantify ITC concentrations in our broccoli samples.

Unfortunately, we discovered early on in the project that the low molar absorptivity of sulforaphane and other ITCs makes direct analysis by HPLC difficult. Even if the concentration of ITCs is amplified in samples by solvent extraction, only samples containing relatively high concentrations of ITCs can be accurately quantified.

To solve this problem, we utilized a method in which ITCs are conjugated to 2-mercaptoethanol before analysis by HPLC (Vermeulen and others 2006). Briefly, food samples are homogenized and first extracted with dichloromethane, and the resulting extract is incubated with a solution of triethylamine and 2-mercaptoethanol in dichloromethane. After the mixture is incubated at room temperature for one hour, the solvent can be removed, and the sample can be reconstituted in methanol before analysis by HPLC. The resulting ITC-mercaptoethanol conjugates have a maximum absorbance wavelength of 271 nm rather than 235 nm, and their UV spectra have a characteristic double peak shape that makes them easier to identify by PDA than unconjugated ITCs (Figure 2.3). Finally, the sensitivity of analyzing ITCs by this method is approximately 10 times greater than analyzing unconjugated ITCs, meaning that lower concentrations of sulforaphane can be quantified. Figure 2.4 shows a chromatogram in which sulforaphane, iberin, and erucin mercaptoethanol conjugates were separated and quantified. All
measurements of sulforaphane discussed below were done by conjugation to 2-mercaptoethanol.
Figure 2.3 Comparison of UV spectra of sulforaphane (top) and sulforaphane-mercaptoethanol conjugate (bottom).
Figure 2.4 Chromatogram showing separation of sulforaphane (SFN), erucin (ERU), and iberin (IBR) conjugated to 2-mercaptoethanol (2-ME) in broccoli sprout homogenate.

To verify the reliability of this method for quantifying ITC concentration in our samples containing broccoli and broccoli sprouts, we performed spike-recovery tests on broccoli homogenates. Known quantities of ITCs produced from standards were added to homogenates of broccoli sprouts, and the homogenates were extracted in the manner described above. Peak areas of the ITC-mercaptoethanol conjugates were compared in the spiked and unspiked samples, and the differences in peak area were compared to the difference expected based on standard curves made from the conjugates. Recovery rates were 95% or above for all ITCs tested, with the exception of allyl ITC (Table 2.1). The low recovery of allyl ITC may be due to its especially high volatility. Since the recovery rate for sulforaphane was so high (99.2%), we felt confident that the mercaptoethanol
extraction method was a reliable tool for quantifying sulforaphane formation in broccoli sprouts.

Table 2.1 Recovery rates of various ITCs when analyzed by the mercaptoethanol conjugation method. Mean±standard deviation

<table>
<thead>
<tr>
<th>ITC Name</th>
<th>Sulforaphane</th>
<th>Iberin</th>
<th>Erucin</th>
<th>Phenethyl ITC</th>
<th>Allyl ITC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recovery Rate (%)</td>
<td>99.2±2.7</td>
<td>94.8±1.5</td>
<td>96.0±6.5</td>
<td>99.8±0.7</td>
<td>28.7±4.3</td>
</tr>
</tbody>
</table>

2.4.2 Effects of 60°C pretreatment on sulforaphane formation in broccoli sprouts

Recent published research indicates that pretreating broccoli sprouts to 60°C for 10 minutes can inactivate ESP while retaining myrosinase activity, increasing the formation of sulforaphane in vitro (Matusheski, Juvik, and Jeffery 2004). Before producing 60°C pretreated freeze-dried broccoli sprout powders, we verified these findings in the lab.

Many publications concerned with ITC formation in broccoli and broccoli sprouts assume that sulforaphane is the only hydrolysis product of glucoraphanin and that full conversion of glucoraphanin to sulforaphane can be achieved by allowing homogenates of raw broccoli sprouts to incubate at 37 or 45°C for a few hours (Shapiro and others 2001; Campas-Baypoli and others 2010). We first noticed a problem with this assumption when the quantity of sulforaphane measured in incubated raw broccoli sprout homogenates was much lower than the quantity of glucoraphanin broccoli sprouts are reported to contain. We found that when broccoli sprouts are steamed and incubated with purified myrosinase, the concentration of sulforaphane in the resulting homogenate is much greater than that of raw homogenates.
After learning about the presence of ESP in broccoli sprouts, we hypothesized that this disparity would be minimized if the sprouts were pretreated to 60°C for ten minutes before homogenization and incubation, as described by Matusheski and others (2006). When compared to raw homogenates, the pretreated broccoli sprout homogenates were found to contain much greater concentrations of sulforaphane (Figure 2.5).

![Sulforaphane concentration in raw and 60°C pretreated broccoli sprout homogenates. Error bars represent standard deviation.](image)

**Figure 2.5** Sulforaphane concentration in raw and 60°C pretreated broccoli sprout homogenates. Error bars represent standard deviation.

### 2.4.3 Utilization of raw daikon radish sprouts as a food grade source of myrosinase

Published results on ITCs and cruciferous vegetables indicate that daikon radish sprouts are an excellent source of natural myrosinase. Before producing our freeze-dried powder of 90% steamed broccoli sprouts and 10% raw daikon radish sprouts, we verified
that raw daikon radish sprouts are as effective as purified myrosinase at converting GLUs in broccoli sprouts to ITCs.

We began by measuring the formation of ITCs in raw broccoli sprout homogenates under incubation conditions described in literature. Publications describing the formation of ITCs in broccoli and broccoli sprouts describe a variety of times and temperatures for incubating broccoli homogenates to fully convert GLUs to ITCs. Methods utilized include incubation at room temperature, 37°C, and 45°C for anywhere from two to eight hours (Shapiro and others 2001; Matusheski, Juvik, and Jeffery 2004; Campas-Baypoli and others 2010). To determine what incubation time and temperature is adequate for full conversion of GLUs to ITCs, we measured the concentrations of ITCs in steamed broccoli sprout homogenates treated with purified myrosinase. Figure 2.6 shows the results of incubating the homogenates at various times and temperatures. We found that incubation at 45°C resulted in greater conversion of glucoraphanin to sulforaphane than incubation at 37° or room temperature and that incubation for two hours does not fully convert glucoraphanin to sulforaphane. For future tests in which full conversion of GLUs to ITCs was required, incubation at 45°C for three to three and a half hours was used.
To test whether raw daikon radish sprouts were as effective as purified myrosinase at converting GLUs to ITCs, we compared the effects of adding raw daikon radish sprouts to steamed broccoli sprout homogenates at a ratio of 90:10 broccoli sprouts to raw daikon sprouts. After incubation, the amount of sulforaphane formed in the homogenate was compared to that formed when purified myrosinase was used. Figure 2.7 shows that full conversion of glucoraphanin to sulforaphane was achieved in both cases, indicating that daikon radish sprouts are a useful source of natural, food grade myrosinase.

Figure 2.6 Concentration of sulforaphane in steamed broccoli sprout homogenates incubated with purified myrosinase at various conditions. Error bars represent standard deviation.
Figure 2.7 Concentration of sulforaphane in steamed broccoli sprout homogenates following incubation with raw daikon sprouts and purified myrosinase, as compared to that in raw broccoli sprout homogenate.

2.4.4 Formation of sulforaphane in soy-tomato juice after addition of broccoli sprout powders

The pungency of ITCs can provide a significant, often undesirable effect on the sensory properties of foods that contain them. Since the manner in which broccoli sprouts are heat-treated has been shown to have an effect on the formation of broccoli sprout homogenates in vitro, we hypothesized that our freeze-dried powders would contribute varying levels of ITCs when added to our soy-tomato juice. Furthermore, we were interested in the extent to which differences in sulforaphane formation in the soy-tomato juice would correlate to the sensory acceptability of the resulting beverages.

To test our hypothesis, we performed an experiment in which we added our four freeze-dried broccoli sprout powders to our soy-tomato juice and measured the formation of sulforaphane over time. After the powders were added to the juice, aliquots were made
and placed into boiling water at varying time points to inactivate myrosinase, halting the formation of ITCs. Samples were then cooled, extracted with dichloromethane, conjugated with 2-mercaptoethanol, and analyzed by HPLC. Figure 2.8 shows the amount of sulforaphane formed in the juice per gram of freeze-dried powder added over time. As predicted, the mixture containing 60P produced the most sulforaphane, followed by RP, and the sample containing SP showed very low levels of sulforaphane, presumably due to the lack of myrosinase. Interestingly, the mixtures containing 60P, RP, and SP showed no identifiable trend with regard to sulforaphane concentration, but the sample containing SDP clearly displayed a gradual increase in sulforaphane concentration over time.

Figure 2.8 Concentration of sulforaphane in soy-tomato juice at various times following the addition of freeze-dried broccoli sprout powders. Error bars represent standard error.
Unexpectedly, we observed that the difference between the amount of sulforaphane formed in the juice containing RP and that containing 60P was much less dramatic than that found when fresh sprouts and 60°C pretreated sprouts were homogenized and incubated. This may indicate that the freeze-drying process or conditions in the soy-tomato juice favor the action of myrosinase over that of ESP. This was unexpected because nitrile formation is favored over ITC formation at lower pH (Matusheski and Jeffery 2001).

Interestingly, when the freeze-dried broccoli sprout powders were analyzed for their content of glucoraphanin, glucoerucin, and glucoiberin, the three most abundant GLUs in broccoli sprouts, much greater concentrations of GLUs were found in the SDP than the 60P or the RP (Table 2.2). These data indicate that GLUs in the broccoli sprouts are likely converted to ITCs during production of the RP and 60P powders. Introducing myrosinase to the powders in the form of raw daikon radish sprouts may prevent the conversion of GLUs to ITCs in the powders before they are mixed into the soy-tomato juice, which may explain why the SDP demonstrated a delayed release of sulforaphane into the beverage whereas the 60P and RP did not.
Table 2.2 Concentrations of glucoraphanin, glucoerucin, and glucoiberin in freeze-dried broccoli sprout powders.

<table>
<thead>
<tr>
<th>Freeze-Dried Broccoli Sprout Powder Analyzed</th>
<th>Average Glucoraphanin Concentration (µmol/g)</th>
<th>Average Glucoerucin Concentration (µmol/g)</th>
<th>Average Glucoiberin Concentration (µmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>90% Steamed Broccoli Sprout plus 10% Raw Daikon Sprout (SDP)</td>
<td>33.9±4.8</td>
<td>15.2±2.1</td>
<td>0.849±.145</td>
</tr>
<tr>
<td>60°C Pretreated (60P)</td>
<td>4.43±0.53 * 10^{-4}</td>
<td>1.79±0.33 * 10^{-4}</td>
<td>Not Detected</td>
</tr>
<tr>
<td>Raw (RP)</td>
<td>8.88±0.02 * 10^{-4}</td>
<td>3.47±0.07 *10^{-4}</td>
<td>Not Detected</td>
</tr>
</tbody>
</table>

The gradual release of sulforaphane in the SDP beverage is promising because it may indicate that the pungent effects of ITCs could be minimized while the healthful benefits of active myrosinase are still retained. Our data indicate that while the mixtures containing 60P and SDP provided similar levels of sulforaphane after 20 minutes, the sulforaphane concentration in the SDP beverage was relatively low through 10 minutes after mixing. Since the beverage would presumably be consumed within 10 minutes after the freeze-dried powders are mixed with the juice, utilizing the delayed sulforaphane release of the SDP could decrease the pungent properties of the juice by limiting sulforaphane formation until after the beverage has been consumed. In other words, our data indicate that the SDP beverage may provide similar health benefits as the 60P beverage without being as pungent.

2.4.5 Sensory properties of various broccoli sprout powders in soy-tomato juice

In the first sensory study performed, subjects were asked to evaluate the sensory properties of beverages consisting of each of the four broccoli sprout powders mixed with previously canned soy-tomato juice at a level of 1% by weight, along with a control of only soy-tomato juice. Subjects were first asked to evaluate the beverages for their
overall acceptability. Figure 2.9 shows the average overall acceptability for each of the five samples tested.

![Bar chart showing overall acceptability of various freeze-dried broccoli sprout powders in soy-tomato juice. Error bars represent 95% confidence interval. Letters represent significant differences at the .05 level.](image)

**Figure 2.9** Overall acceptability of various freeze-dried broccoli sprout powders in soy-tomato juice. Error bars represent 95% confidence interval. Letters represent significant differences at the .05 level.

The data on overall acceptability show that all samples containing broccoli sprout powder were significantly less acceptable than the control of only soy-tomato juice. This indicates that the amount of broccoli sprout powder in the juice has a greater effect on acceptability than the type of powder added. The control had an overall acceptability of 6.33 (slightly liked), whereas the samples containing broccoli sprout powder had acceptability values ranging from 4.08 (disliked lightly) to 4.88 (neither liked nor disliked).
When comparing the samples containing broccoli sprout powder, the SDP, SP, and 60P samples had very similar acceptability values (4.86, 4.88, and 4.76, respectively), whereas the RP sample had a lower acceptability of 4.08 (although this difference was not significant at the .05 level). Since the SDP and 60P samples are thought to have the greatest cancer preventative potential, due to the presence of active myrosinase and lack of ESP, the data for overall acceptability indicate that these samples may be more promising than the SP or RP samples.

Subjects were next asked to evaluate the subjects for their amount of broccoli flavor and pungency/spiciness on a 5-point JAR scale, where 1 represents way too little and 5 represents way too much. Data for these tests is illustrated in figure 2.10. With regard to pungency, JAR values ranged from 2.76 (control) to 3.64 (RP and 60P). Samples containing SP and SDP were intermediate, scoring 3.29 and 3.49, respectively. These results indicated that the samples varied somewhat in their level of pungency and that the SDP may provide a more acceptable level of pungency than the 60P. A Tukey’s Honestly Significant Difference (HSD) test indicates that the control was significantly less pungent than the samples containing broccoli sprouts, but differences between the remaining samples were too subtle to be significant at the .05 level.
Figure 2.10  Just About Right (JAR) scores for amount of pungency and broccoli flavor of various freeze-dried broccoli sprout powders in soy-tomato juice. Error bars represent 95% confidence interval. a–c Letters represent significant differences at the .05 level.

With regard to amount of broccoli flavor, the samples showed a slightly larger range (2.51 to 3.93 for control and RP, respectively). Subjects indicated that the control of soy-tomato juice did not contain enough broccoli flavor, whereas the sample containing RP provided slightly too much broccoli flavor. The samples containing SP, SDP, and 60P provided an intermediate amount of broccoli flavor that was not significantly different at the .05 level. These results indicate that the samples containing SP, SDP, and 60P were generally more preferred in terms of the amount of pungency and broccoli flavor they provided than either the control or the sample containing RP.

Finally, subjects ranked the samples based on their overall preference. Figure 2.11 illustrates the rank totals for each sample, where a lower total rank score represents a more favored sample. As predicted, the control was clearly favored over the samples.
containing broccoli sprout powders. The rank total followed a very similar pattern as the overall acceptability, with the sample containing SP being most favored followed by SDP, 60P, and RP, respectively. Although the differences are not significant, these results, when taken in conjunction with the overall acceptability values and comments provided by subjects, indicate that the samples containing SP and SDP were slightly favored over the sample containing 60P and somewhat favored over the sample containing RP. Since the SDP has the added benefit over the SP of containing active myrosinase activity, we concluded that the SDP may represent the best compromise between cancer preventative potential and sensory acceptability, although the 60P sample would be worth investigating further as well.
2.4.6 Sensory effects of varying concentration of SDP in soy-tomato juice

In the second sensory study, the SDP was added to a newly canned soy-tomato juice at concentrations of 0, .25, .5, and 1% and to the previously canned soy-tomato juice at a level of 1%. The addition of the final sample allows for comparison of the results of the second study with those of the first, despite the difference in juice. Subjects were asked to evaluate the samples for overall acceptability, amount of broccoli flavor, and amount of pungency/spiciness and then asked to rank the samples in order of overall preference, just as before.

We chose to use newly canned soy-tomato juice in the second sensory study because many subjects in the initial study indicated that the juice had an off-putting, overly strong flavor. We suspected this may have been due to the age of the juice, as it had been in storage for over a year, and we also suspected that the previously canned juice may have been formulated with too much salt, resulting in a metallic flavor.
Furthermore, we hypothesized that the flavor of the juice could have a significant effect on the amount of broccoli sprout powder that could be added to it before the beverage became unacceptable. In other words, we thought that using newly canned juice would give us a better idea of the sensory properties of the final product that will eventually be produced in the future. To allow the data from the second sensory experiment to be compared to the data from the initial experiment, we included a sample of 1% SDP in the previously canned juice, which was one of the samples used in the initial experiment.

Before the soy-tomato juice was newly canned, the stability of the isoflavones in the soy isoflavone concentrate was confirmed by measuring the concentrations of five isoflavones in the concentrate. After the juice was canned, it was also extracted for isoflavones, and the concentrations of the isoflavones in the juice was compared to those predicted based on the proportion of soy isoflavone concentrate in the juice. Figures 2.12 and 2.13 show that the isoflavones in the concentrate were stable throughout the canning process.
Figure 2.12 Concentrations of glycitin, daidzein, and genistein in soy-tomato juice. Error bars represent standard deviation.

Figure 2.13 Concentrations of daidzin and genistin in soy-tomato juice. Error bars represent standard deviation.
Once the newly canned soy-tomato juice was canned, we were able to assess the sensory properties of the SDP in the juice at varying concentrations. Figure 2.14 displays the average values for overall acceptability for the beverages. The sample containing only juice had the highest overall acceptability (5.68), and the addition of broccoli sprout powder decreased the overall acceptability in a dose-dependent manner. The samples containing 1% SDP, both in the new and previously canned juice, had significantly lower acceptability values than the juice without SDP, but the samples containing .25 and .5% were not significantly different from the juice alone, based on a Tukey’s HSD test. Both the .25 and .5% samples had acceptability values near 5, indicating that subjects neither liked nor disliked them, whereas the subjects slightly disliked the samples containing 1% powder. These results indicate that a concentration of approximately .5% SDP can be added to the juice without making the juice unacceptable in terms of overall acceptability.

![Figure 2.14](image-url)

**Figure 2.14** Average overall acceptability of freeze-dried steamed broccoli sprout plus raw daikon sprout powder (SDP) at various concentrations in soy-tomato juice. Error bars represent 95% confidence interval. \(^{a-c}\)Letters represent significant differences at the .05 level.
Subjects were next asked to evaluate the samples on their amount of broccoli flavor and pungency on a 5-point JAR scale (Figure 2.15). In both cases, the addition of SDP increased the JAR score in a dose-dependent manner. With regard to amount of broccoli flavor, the sample of juice only was judged to have slightly too little (2.50), whereas both samples containing 1% SDP were judged to have slightly too much (3.91 and 3.56 for the new and old juices, respectively). Both the samples containing .25 and .5% SDP had a JAR value not significantly different from 3, indicating that these concentrations provide the right amount of broccoli flavor to the juice.

Figure 2.15  Just About Right (JAR) scores for amount of pungency and broccoli flavor in soy-tomato juice with freeze-dried steamed broccoli sprout and raw daikon radish sprout powder (SDP) at various concentrations. Error bars represent 95% confidence interval. a-d Letters represent significant differences at the .05 level.

When subjects were asked to evaluate the beverages for their amount of pungency, a similar result was found, with the exception that the sample of 1% SDP in
the previously canned juice was judged to be by far the most pungent. This is likely due to the strong flavor and bitterness of the previously canned juice, and it may explain why the sample of 1% SDP in the newly canned juice was judged to have more broccoli flavor. Both the samples containing .25 and .5% SDP had a JAR value not significantly different from 3, indicating, as before, that these concentrations result in a good amount of pungency to the juice.

Finally, subjects ranked the samples in order of their overall preference. As shown in figure 2.16, the order of preference was slightly different from the order of overall acceptability score. The sample of .25% SDP in the newly canned juice was the most favored, and the sample of .5% SDP had a nearly identical score to the sample of juice only. In contrast, both samples containing 1% SDP had significantly higher overall rank scores. These data, when taken in consideration with the overall acceptability and JAR scores, indicate that a concentration of .5% SDP can be added to the powder without significantly hampering the overall sensory properties of the beverage.
Observations taken from the comments the subjects provided and comparison of the data for the samples containing the newly canned and previously canned juices indicated a few interesting insights. Although the newly canned juice had a fresher flavor and many subjects indicated that the sample containing the previously canned juice was overly bitter or pungent, the overall acceptability value for the newly canned juice was actually lower than that of the previously canned juice in the first study. In fact, 21% of participants listed the sample containing 1% SDP in the previously canned juice as their most favored, whereas 51% ranked this sample fourth or fifth. The previously canned juice was polarizing in this way. Based off comments the subjects provided, the thicker texture and saltiness of the previously canned juice was preferred by some. In the future, we may want to investigate the effects of increasing the saltiness and thickness of the soy-tomato juice to better complement the addition of broccoli sprout powder.
Ultimately, this may allow us to improve the sensory characteristics of the juice and/or increase the content of broccoli sprout powder that is added.

2.5 Conclusions

Our results have confirmed published findings that mild heat treatments can increase the formation of sulforaphane in broccoli sprout homogenates in vitro. Furthermore, we demonstrated how conjugating ITCs to 2-mercaptoethanol can greatly improve the sensitivity of ITC quantification in food samples. Using these findings, we produced four freeze-dried broccoli sprout powders that can be mixed with our soy-tomato juice to create four novel soy-tomato-broccoli sprout beverages. By measuring the formation of sulforaphane in the soy-tomato juice after the addition of our freeze-dried broccoli sprout powders, we were able to demonstrate how the manner in which broccoli sprouts are heat-treated can affect the extent and rate of sulforaphane formation in our soy-tomato juice. Interestingly, the mixture containing SDP showed a delayed release of sulforaphane, indicating that the SDP beverage may provide the healthful benefits of active myrosinase without providing the same pungency as the 60P or RP beverages.

When we combined our various freeze-dried broccoli sprout powders with soy-tomato juice to create four new prototypes of a soy-tomato-broccoli sprout beverage, subjects indicated that the samples containing SDP and 60P were not significantly different in terms of sensory properties from the sample containing only SP without daikon sprout. Since the samples containing SDP and 60P are hypothesized to provide a greater cancer preventative potential than the sample containing SP, due to the presence of active myrosinase and lack of ESP, we concluded that either would be promising to investigate further. Since the sample containing SDP showed slightly better sensory
results than the sample containing 60P, we chose to use SDP in our second sensory experiment.

Next, we performed a second study in which we added SDP to a newly canned soy-tomato juice at varying concentrations. We found that a concentration of approximately .5% SDP is acceptable to add to the new juice without significantly hampering its sensory properties. Therefore, the addition of approximately .5% SDP to the soy-tomato juice represents the current best prototype of our new soy-tomato-broccoli sprout beverage that provides the best compromise between maximizing ITC delivery and providing sensory acceptability.

Moving forward, efforts should be made to further improve the sensory properties of the juice, as this would not only make it more pleasing to consume, but also allow for increased concentrations of broccoli sprout powder to be added to the beverage, increasing its cancer preventative potential. Subjects in the second sensory test indicated that a thicker texture is desired, which may be achieved by homogenizing the juice to a greater extent or by adding thickening agents. Saltiness, sourness, and temperature of the beverage may also play an important role in the overall acceptability of the juice, and these properties could be easily modified and optimized using further sensory testing.

In addition, the broccoli sprout powder prototypes we used were developed based on the assumption that since modulating the activity of ESP and myrosinase in the sprouts affects the formation of ITCs in vitro, this will also affect the bioavailability of the ITCs; however, this has yet to be verified. An interesting biological experiment would be to compare ITC absorption after subjects have consumed equal weights of raw, steamed, and 60°C pretreated broccoli sprouts, as well as steamed broccoli sprouts with
raw daikon radish sprouts, in order to determine the extent to which ESP activity affects ITC bioavailability. If our hypothesis is false and it is found that inactivating ESP has little effect on absorption, it will be interesting to investigate why the body favors formation of sulforaphane over sulforaphane nitrile, especially considering that consuming cruciferous vegetables with enzymatic activity intact increases ITC absorption.
REFERENCES


Waterman SR, Small PL. 1998. Acid-sensitive enteric pathogens are protected from killing under extremely acidic conditions of pH 2.5 when they are inoculated onto certain solid food sources. Appl Environ Microbiol 64:3882-6.


APPENDIX A: SENSORY EVALUATION BALLOT PROVIDED TO PARTICIPANTS
Welcome to Sensory Testing!
Melody Leidheiser, Sensory Evaluation Program Coordinator

This study is designed to evaluate the sensory acceptance of several soy-tomato-broccoli beverages. In this study, you will be presented with five samples of soy-tomato-broccoli juice and asked to assess their flavor. At the end, you will be asked a few demographic questions such as gender and age. You may not participate if you are under the age of 18, or if you are allergic to soy, tomato, or broccoli products.

Your answers will be entered directly into the computer using a mouse and keyboard. This has been estimated to take about 15 minutes, but you may take as long as you need. Your responses will in no way be linked to your identity. For participating in the test, you will be compensated with a $5 Target Gift Card. If you have any questions, please feel free to ask the attendant at any time.

Please LOOK AT the first sample ### and answer the following question…

Rate the ACCEPTABILITY OF THE FLAVOR of this sample from 1 (dislike extremely) to 9 (like extremely).

1_________________________9

Dislike Extremely
Like Extremely

Rate the AMOUNT OF BROCCOLI FLAVOR in this sample from 1 (way too little) to 5 (way too much).

1_________________________3_________________________5

Way Too Little Just About Right Way Too Much

Rate the PUNGENCY/SPICINESS of this sample from 1 (way too little) to 5 (way too much).

1_________________________3_________________________5

Way Too Little Just About Right Way Too Much

** Please feel free to comment on the sample you just tasted by using the keyboard or mouse.

Repeat with the remaining 4 samples (###, ###, ###, ###) using the same question format.

Please rank the following samples (###, ###, ###, ###) by overall acceptability from BEST to WORST:
FINALLY:

Please ANSWER the following demographic questions.

Please choose one box below to indicate your GENDER.

- Male
- Female

Please choose one box below to indicate your AGE CATEGORY.

- 18 – 20 years
- 21 – 25 years
- 26 – 35 years
- 36 – 45 years
- 46 – 55 years
- 56 – 65 years
APPENDIX B: WRITTEN CONSENT FORM PROVIDED TO SENSORY PARTICIPANTS
The Ohio State University Consent to Participate in Research

Study Title: Consumer Acceptance of Soy-Tomato-Broccoli Beverages

Researcher: Dr. Steven J. Schwartz

This is a consent form for research participation. It contains important information about this study and what to expect if you decide to participate.

Your participation is voluntary.

Please consider the information carefully. Feel free to ask questions before making your decision whether or not to participate. If you decide to participate, you will be asked to sign this form and will receive a copy of the form.

Purpose:

The purpose of this study is to determine the consumer acceptability of several soy-tomato-broccoli beverages to assist in formulation.

Procedures/Tasks:

You will be given five samples of soy-tomato-broccoli juice and asked to evaluate them on their overall acceptability, amount of broccoli flavor, and amount of pungency. You will then be asked to rank the samples in order of your preference. Finally, you will be asked to complete a short demographic survey.

Duration:

The study will last approximately 15 minutes. You may leave the study at any time. If you decide to stop participating in the study, there will be no penalty to you, and you will
not lose any benefits to which you are otherwise entitled. Your decision will not affect your future relationship with The Ohio State University.

**Risks and Benefits:**

The samples you will be given contain tomato, soy, and broccoli-based ingredients. If you are allergic to these foods, you may not participate in the study. Other risks should be no greater than you experience in daily life. If you suffer from a medical condition for which you limit your intake of soy, tomato, broccoli, or sodium, you are discouraged but not prohibited from participating.

**CONFIDENTIALITY:**

Efforts will be made to keep your study-related information confidential. However, there may be circumstances where this information must be released. For example, personal information regarding your participation in this study may be disclosed if required by state law. Also, your records may be reviewed by the following groups (as applicable to the research):

- Office for Human Research Protections or other federal, state, or international regulatory agencies;
- The Ohio State University Institutional Review Board or Office of Responsible Research Practices;
- The sponsor, if any, or agency (including the Food and Drug Administration for FDA-regulated research) supporting the study.

**Incentives:**

You will be given a $5 gift card to Target for participating in the study. You do not need to fully complete the study to receive the incentive.

**PARTICIPANT RIGHTS:**

You may refuse to participate in this study without penalty or loss of benefits to which you are otherwise entitled. If you are a student or employee at Ohio State, your decision will not affect your grades or employment status.
If you choose to participate in the study, you may discontinue participation at any time without penalty or loss of benefits. By signing this form, you do not give up any personal legal rights you may have as a participant in this study.

CONTACTS AND QUESTIONS:

The attendant currently administering this test is Ryan T. Riddle. If you have questions related to the study, you may ask him personally or contact him by phone (832 247-0607) or by e-mail (Riddle.87@osu.edu).

For questions related to the study, you may contact the principal investigator, Dr. Steven J. Schwartz, by phone (614 292-2934) or by e-mail (Schwartz.177@osu.edu).

For questions about your rights as a participant in this study or to discuss other study-related concerns or complaints with someone who is not part of the research team, you may contact Ms. Sandra Meadows in the Office of Responsible Research Practices at 1-800-678-6251.

If you are injured as a result of participating in this study or for questions about a study-related injury, you may contact the principal investigator, Dr. Steven J. Schwartz, by phone (614 292-2934) or by e-mail (Schwartz.177@osu.edu).

SIGNING THE CONSENT FORM

I have read (or someone has read to me) this form and I am aware that I am being asked to participate in a research study. I have had the opportunity to ask questions and have had them answered to my satisfaction. I voluntarily agree to participate in this study.

I am not giving up any legal rights by signing this form. I will be given a copy of this form.
<table>
<thead>
<tr>
<th>Printed name of subject</th>
<th>Signature of subject</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AM/PM</td>
</tr>
<tr>
<td></td>
<td>Date and time</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Printed name of person authorized to consent for subject (when applicable)</th>
<th>Signature of person authorized to consent for subject (when applicable)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AM/PM</td>
</tr>
<tr>
<td></td>
<td>Date and time</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Relationship to the subject</th>
<th>Date and time</th>
</tr>
</thead>
</table>

**Investigator/Research Staff**

I have explained the research to the participant or his/her representative before requesting the signature(s) above. There are no blanks in this document. A copy of this form has been given to the participant or his/her representative.

<table>
<thead>
<tr>
<th>Printed name of person obtaining consent</th>
<th>Signature of person obtaining consent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AM/PM</td>
</tr>
<tr>
<td></td>
<td>Date and time</td>
</tr>
</tbody>
</table>
APPENDIX C: APPROVAL LETTERS FOR EXEMPTION FROM IRB REVIEW
May 2, 2011

Protocol Number: 2011E0193

Protocol Title: CONSUMER ACCEPTANCE AND THRESHOLD CONCENTRATION OF BROCCOLI SPROUT POWDERS IN SOY-TOMATO JUICE. STEVEN SCHWARTZ, FOOD SCIENCE & TECHNOLOGY

Type of Review: Request for Exempt Determination

Dear Dr. Schwartz,

The Office of Responsible Research Practices has determined the above referenced protocol exempt from IRB review.

Date of Exempt Determination: 4/26/2011
Qualifying Exemption Category: 6

Please note the following:

- Only OSU employees and students who have completed CITI training and are named on the signature page of the application are approved as OSU Investigators in conducting this study.
- No changes may be made in exempt research (e.g., personnel, recruitment procedures, advertisements, instruments, etc.). If changes are needed, a new application must be submitted.
- Per university requirements, all research-related records (including signed consent forms) must be retained and available for audit for a period of at least three years after the research has ended.
- It is the responsibility of the investigator to promptly report events that may represent unanticipated problems involving risks to subjects or others.

This determination is issued under The Ohio State University's OHRP Federalwide Assurance #0006378. All forms and procedures can be found on the OERP website – www.orrp.osu.edu. Please feel free to contact the OERP staff or contact listed below with any questions or concerns.

Cheri Petey, MA, Certified IRB Professional
Senior Protocol Analyst—Exempt Research

Office of Responsible Research Practices
Ohio State University
1960 Kenny Road
Columbus, OH 43210
phone: 614.688.0369
fax: 614.688.6346
email: petey.6@osu.edu
October 14, 2011

Protocol Number: 2011E0609
Protocol Title: CONSUMER ACCEPTANCE OF SOY-TOMATO-BROCCOLI BEVERAGES: PART II, STEVEN SCHWARTZ, FOOD INDUSTRY CENTER
Type of Review: Request for Exempt Determination

Dear Dr. Schwartz,

The Office of Responsible Research Practices has determined the above referenced protocol exempt from IRB review.

Date of Exempt Determination: 10/11/2011
Qualifying Exemption Category: 6

Please note the following:

- Only OSU employees and students who have completed CITI training and are named on the signature page of the application are approved as OSU Investigators in conducting this study.
- No changes may be made in exempt research (e.g., personnel, recruitment procedures, advertisements, instruments, etc.). If changes are needed, a new application must be submitted.
- Per university requirements, all research-related records (including signed consent forms) must be retained and available for audit for a period of at least three years after the research has ended.
- It is the responsibility of the Investigator to promptly report events that may represent unanticipated problems involving risks to subjects or others.

This determination is issued under The Ohio State University’s OHRP Federalwide Assurance #00006378. All forms and procedures can be found on the ORRP website – www.orrp.osu.edu. Please feel free to contact the ORRP staff contact listed below with any questions or concerns.

Cheri Pettry, MA, Certified IRB Professional
Senior Protocol Analyst—Exempt Research
Office of Responsible Research Practices
Ohio State University
1960 Kenny Road
Columbus, OH 43210
phone: 614.688.0389
fax: 614.688.0386
email: pettry.66@osu.edu
APPENDIX D: RECRUITMENT LETTER FOR SENSORY STUDIES
The Sensory Analysis Laboratory, Food Science & Technology

Date: May 21, 2011
Time: By Appointment: 10:00 am – 4:00 pm.

Please respond to www.foodindustries.osu.edu to sign up for a 30 minute period by Tuesday, December 14.

Place: Parker Building 122 Sensory Laboratory, 2015 Fyffe Road, Columbus OH building 64.
Perk: $5 Target Gift Card

Your help is sought to evaluate several soy-tomato-broccoli juices. In this study, you will be presented with five samples of soy-tomato-broccoli juice in a random order. You will be asked to evaluate them based on the acceptability of their flavor. You will judge each sample using easily understood prompts from our computer display. Finally, you will answer a few demographic questions such as gender and age, but your identity remains confidential.

Anyone who is allergic to soy, tomato, or broccoli products or is under 18 years of age may not participate.

Your answers go directly into a computer using a mouse and keyboard. This will likely take no more than 15 minutes but there is no time limit. Your participation is voluntary and you may refuse to answer any question. Your responses are not linked to your identity. For participating, you will be given a
$5.00 gift card to Target.

If you have questions you may contact Melody Leidheiser at leidheiser.10@osu.edu or Ryan T. Riddle at Riddle.87@osu.edu. Everyone working on this project is affiliated with The Ohio State University.

Thank you.
APPENDIX E: STANDARD CURVES
Figure E.1 Standard curves of sulforaphane-mercaptoethanol conjugate used to quantify sulforaphane concentration in broccoli sprout homogenates and soy-tomato juice.
Figure E.2  Standard curves of iberin- and erucin-mercaptoethanol conjugates used to quantify iberin and erucin concentrations in broccoli sprout homogenates.
Figure E.3 Standard curves of allyl ITC- and phenethyl ITC-mercaptoethanol conjugates used to quantify allyl ITC and phenethyl ITC in spike-recovery experiment in broccoli sprout homogenates.
Figure E.4 Standard curves of daidzin, genistin, and glycitin used to quantify concentrations of these isoflavones in soy-tomato juice.
Figure E.5 Standard curves for daidzein and genistein used to quantify concentrations of these isoflavones in soy-tomato juice.

Figure E.6 Standard curve for all-trans-lycopene used to quantify lycopene concentration in soy-tomato juice.