

# **The Development of a Tomato-Soy-Arugula Seed Beverage for Prostate Cancer Clinical Trial**

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## **ABSTRACT**

The relationship between tomato, soy, and cruciferous vegetables with lower risk of prostate cancer has been investigated in several prospective epidemiological studies. It is hypothesized that the combination of these foods reduces the risk of prostate cancer to a greater extent than any single food alone. Cruciferous vegetables such as arugula are rich in a group of compounds called glucosinolates. Upon chewing or tearing of the plant, the endogenous plant enzyme myrosinase is released and hydrolyzes the glucosinolates to isothiocyanates (ITCs). ITCs are bioactive, and have been shown to be chemoprotective in animal and human studies. However, ITCs are not stable in processed food products, and to deliver high levels of ITCs in a food product, they must be converted from glucosinolates immediately before consumption. The purpose of this study is to develop a tomato-soy-arugula seed beverage with optimized delivery of ITCs. The ITCs erucin and sulforaphane were quantified in the tomato-soy-arugula seed beverage under different temperatures, hydrolysis times, pHs, and food matrixes on a C18 column with an optimized HPLC analytical method. By optimizing the time, temperature, matrixes and pH conditions of the tomato-soy-arugula seed beverage, we were able to recover

57% erucin and (67mmol/kg) and 76% sulforaphane (16.4mmol/kg) compared to the control (water). In conclusion, the tomato-soy-arugula seed beverage delivered sufficient doses of ITCs and can be used in future prostate cancer clinical trials.

## INTRODUCTION

Prostate cancer is the second leading cause of cancer death in American men. In 2014, around 233,000 new cases and 29,480 deaths of prostate cancer were documented (Siegel et al., 2014). Although medical advances have reduced the mortality rate, those cured by early detection and treated with prostatectomy, radiation, brachytherapy, cryotherapy or other modalities, can experience lifelong sexual dysfunction and/or urinary incontinence. Efforts to define preventive strategies, reduce the risk of recurrence after initial therapy, and improve therapy are critically needed.

It is our opinion that diet and nutritional interventions may play a role in prostate cancer prevention. History indicates that plants have been used to treat various kinds of diseases, from minor conditions to chronic diseases (McDanell et al., 1982; Steinmetz and Potter, 1996). Several epidemiological studies, which examine large populations over time to gain an understanding of the relationship between specific diets and health benefits, have indicated that high consumption of cruciferous vegetables, such as broccoli and arugula, can reduce risk of multiple cancers (Higdon et al., 2007). Vegetables in this group are rich in nutrients including vitamins C, E, and K, folates, and minerals. Cruciferous vegetables are also rich sources of several different classes of phytochemicals including carotenoids and glucosinolates. Glucosinolates, largely present in cruciferous vegetables and not in other classes of vegetables,

can be further broken down into isothiocyanates (ITC) when the plant's cell structure is broken and the endogenous plant enzyme myrosinase (a thioglucosidase) is released. In addition, ITCs are hypothesized to be the main chemopreventive agents (Zhang et al, 1994). ITCs are well absorbed in humans – a well homogenized crucifer plant allowing optimized contact with myrosinase results in 80% or more absorption of the ITCs (Fahey, 2012).

Besides the benefit of cruciferous vegetables, multiple studies (epidemiologic, clinical, animal model and cell culture studies) on soy and tomato products support that a rich diet in both foods can modulate prostate cancer risk (Clinton et al, 1998; Lee et al, 2003). In a human study, a combination of both soy and tomato has been shown to modulate prostate cancer more efficiently compare to either food alone (Grainger et al, 2008), and in an animal model, the combination of tomato and broccoli also was more effective than either food alone at slowing prostate tumor growth (Canene-Adams et al, 2007). Thus, it is advantageous to consume a diet that includes the combination of these foods and their bioactive constituents.

However, some difficulties exist in the development of a food beverage that contains ITCs. Once myrosinase is released and glucosinolates are converted to ITCs, the ITCs are unstable and can be lost quickly. In addition, creating a food product rich in glucosinolates is less beneficial for health. Glucosinolates are not bioactive in the human body until converted into ITCs. While the conversion to ITCs can be driven by myrosinase in a food product, human gut microbes also have thioglucosidase activities and can convert intact glucosinolates to ITCs after consumption (Fahey et al., 2001). However, consumption of intact glucosinolates versus ITCs results in lower absorption of ITCs and lower *in vivo* biological activity (Clarke et al, 2011). Thus, the development of a functional beverage that directly delivers sufficient levels of ITCs is both technically challenging and important for health.

The goal of this study was to develop a palatable functional beverage that combines tomato, soy, and a cruciferous vegetable and maximizes the delivery of cruciferous vegetable chemopreventive components, for use in future clinical trials. We use the “activation in place” technique, in which the glucosinolates are hydrolyzed to ITCs immediately before consumption.

## MATERIALS AND METHODS

**Chemicals.** HPLC (high performance liquid chromatography) grade solvents (Fisher Scientific; Pittsburgh PA) were used for all analyses. 2-mercaptoethanol and formic acid were purchased from Sigma-Aldrich (St.Louis, MO). Triethylamine was obtained from EMD Chemicals (Gibbstown, NJ). D,L-sulforaphane standards were purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). Erucin standards were obtained from LKT Laboratories (St. Paul, MN).

**Materials.** The tomato juice (Campbell’s), broccoli sprouts, arugula leaves and olive oil (Bertolli Extra Light Tasting Olive Oil) were obtained from the local grocery store. Edible soy isoflavone (Solgen 40S) was provided by CHS (Inner Grove Heights, MN). Untreated arugula seeds were acquired from a local farm (The Chef’s Garden, Huron, OH).

**Analysis of ITCs in Cruciferous Vegetables.** ITC formation from plants was measured using the methods described by Vermeulen et al. (2006), with minor modifications. Broccoli sprouts, arugula leaves, and arugula seeds were ground separately using a mortar and pestle, and 25 mg of each were measured into glass vials. 5 mL of HPLC grade water was added into the vial at temperature of 45°C for 2 hr to allow sufficient glucosinolate hydrolysis. Next, 10 mL dichloromethane was added and the mixture was mechanically shaken for 20 minutes and

centrifuged at 1200xg for 10 min, followed by the removal of the dichloromethane layer. The extraction process was repeated by adding another 10 mL of dichloromethane, and the final extracts were combined, creating approximately 20 mL of extract. A 1 mL aliquot was mixed with 200  $\mu$ L conjugating reagent (20 mM triethylamine and 200 mM 2-mercaptoethanol in dichloromethane). The mixture then was incubated at 30°C for 60 min and dried under gaseous nitrogen. Samples were reconstituted in 1:1 water: acetonitrile, then passed through 0.45  $\mu$ m nylon filters into HPLC vials. Separation and quantification of the 2-mercaptoethanol conjugate of sulforaphane and erucin was performed on a Waters 2695 with a Waters 996 photo diode array detector. 10  $\mu$ L of the sample was injected onto a C18 column (3.5  $\mu$ m, 4.6 x 75 mm) on a linear gradient, consisting of solution A (0.1% formic acid) and solution B (0.1% formic acid in acetonitrile). Initial conditions of 0% B increased to 50% B over 15 minutes, and returned to 0% after 15 minutes. Both the 2-mercaptoethanol conjugated sulforaphane and erucin in broccoli sprouts, arugula leaves, and arugula seeds were quantified by creating a standard curve using sulforaphane and erucin standards conjugated with 2-mercaptoethanol.

**Temperature, Time, Matrix and pH dependence of ITC formation from arugula seeds.** To identify the optimal hydrolysis temperature, the above method was modified and ground arugula seed (25 mg) was hydrolyzed in water at various temperatures (25, 40, 50, 60 °C) for 2 hr and sulforaphane and erucin analyzed with HPLC. Once the optimal temperature was identified, the ground seeds were hydrolyzed in water for different durations (0.5, 1, 3, 5, 10, 15 min) at this temperature and sulforaphane and erucin were again analyzed. After the optimal time and temperature conditions for ITC formation was determined, arugula seeds were then hydrolyzed in the tomato-soy beverage matrix to determine recovery rate. The tomato-soy beverage has a natural pH of 4.2, while it is known that myrosinase has peak activity at a neutral pH. Phosphate

buffer was used to adjust the beverage to approximately neutral (pH 6.8), and the tomato-soy-arugula seed beverages at pH 4.2 and pH 6.8 were analyzed for ITC formation using the same method above.

**Statistical Analysis.** All experiments were performed in triplicate. Statistical analysis was performed by pair t-test and one-way ANOVA using Tukey's family comparisons using IBM SPSS Statistics 22 (IBM Corporation, Armonk, NY). A *P* value of <0.05 was considered significant.

## RESULTS AND DISCUSSION

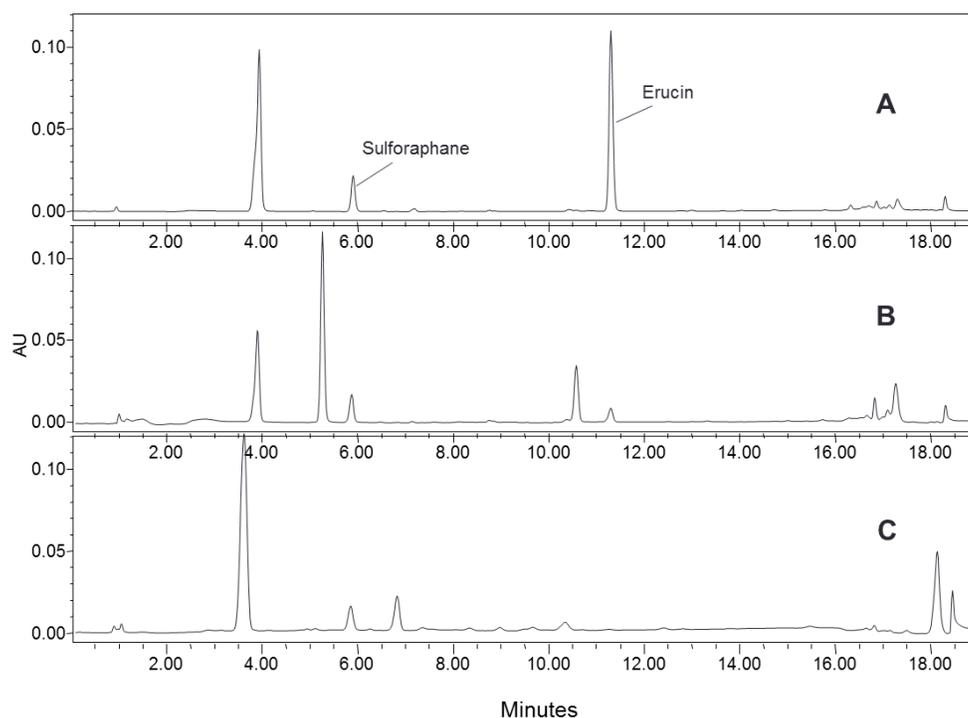
The focus of this study was to optimize the conditions to maximize ITC formation in a tomato-soy-cruciferous vegetable beverage. The first objective was to identify a cruciferous vegetable that will result in high concentrations of chemopreventive components when added to the tomato-soy beverage. Formation of sulforaphane was similar in the three materials chosen (broccoli sprout, arugula leaves, and arugula seeds) ( $P>0.05$ ), while formation of erucin was greatest in arugula seeds, followed by arugula leaves, and lowest in broccoli sprouts ( $P<0.05$ ) (Fig 1). This is likely because arugula seeds are more concentrated in the parent glucosinolates glucoraphanin and glucoerucin compared to both arugula leaves and broccoli sprouts (unpublished preliminary data).

The second objective was to identify the optimum temperature and time for ITC (sulforaphane and erucin) formation following glucosinolate hydrolysis of arugula seeds. Since ITCs are not stable, they are not suited for a product with a long shelf-life. Thus, we created a product in which glucosinolates are hydrolyzed to ITCs immediately before consumption. While

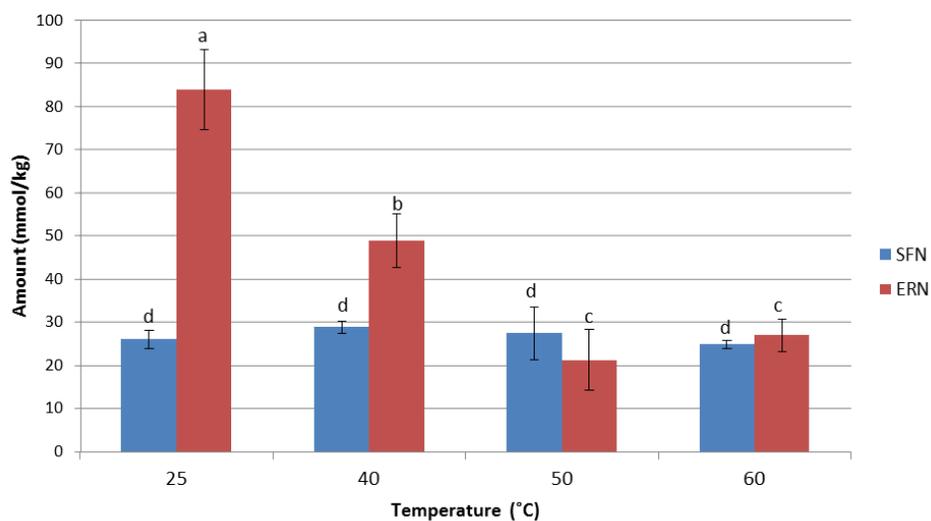
published analytical methods typically use a warm temperature (45°C) for glucosinolate hydrolysis, we examined a more moderate temperature because for a consumer friendly beverage, it is not feasible to heat the tomato-based beverage before consumption. Similarly, while published analytical methods typically allow 2 hr for glucosinolate hydrolysis, we investigated shorter hydrolysis times because 2 hr is not feasible for a consumer product. We examined the technique of “activation in place,” in which the ITCs are formed immediately before consumer consumption. This information gives insight into the rate of ITC formation and myrosinase stability under different conditions. The optimal temperature and hydrolysis time at which erucin formation occurs in arugula seeds lies in room temperature (25 °C) (Fig.2) and between hydrolysis times of 0.5-5 minutes (Fig.3), while sulforaphane formation was not impacted by the different hydrolysis temperatures and times. A 2 hr hydrolysis time was unnecessary. After optimization of the conditions, sulforaphane and erucin are formed in the arugula seed ratio as 1: 5.8 with sulforaphane being  $21.7 \pm 1.1$  mmol/kg and erucin being  $126.8 \pm 10.6$  mmol/kg fresh weight. In addition, 57% of erucin (67mmol/kg) and 76% of sulforaphane (16.4mmol/kg) was retained in the tomato-soy matrix compared to those formed in the control (water) (Fig.4). It is known that myrosinase is more active at neutral pH (Higdon et al., 2007). Due to the low pH (4.2) of tomato-soy beverage, adjustment of the beverage to pH 6.8 elevates the ITCs formation (Fig.5). These results show that it is possible to deliver ITCs from ground arugula seeds in a tomato-soy beverage by hydrolyzing glucosinolates immediately before consumption. While significant, the low pH of the tomato-soy beverage still allowed both sulforaphane and erucin formation. The results of this study show the optimal conditions for bioactive compound formation and that a functional tomato-soy-arugula seed beverage can be of use in a future prostate cancer clinical trial.

## CONCLUSION

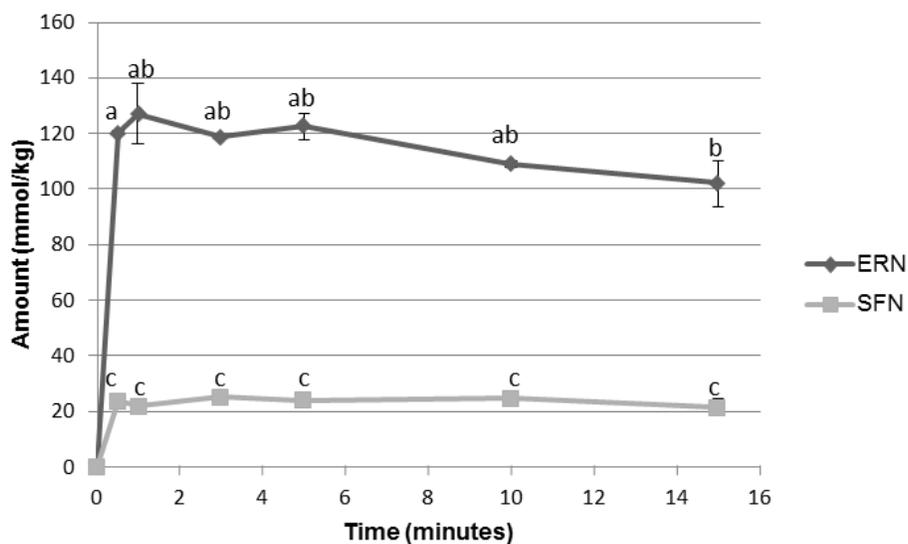
ITC delivery was maximized in the developed tomato-soy-arugula seed beverage. This study supports use of this novel beverage in a future clinical trial to investigate the relationship between consumption of the combined three elements (tomato, soy and arugula) with prostate cancer prevention. Future study is needed on the acceptability of this beverage as a health-promoting product for cancer prevention.



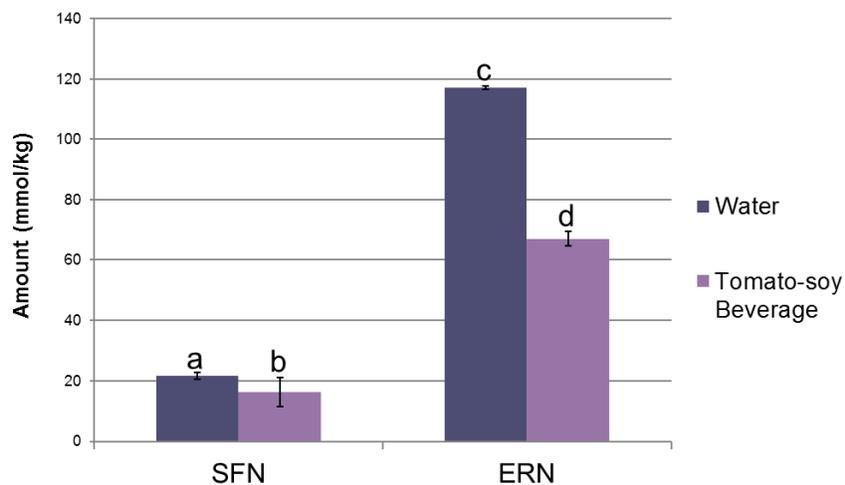
**Fig 1.** Chromatograms of the different compositions found in broccoli sprout, arugula leaves, and arugula seeds. (A) Arugula seeds. (B) Arugula leaves. (C) Broccoli sprouts. The amount of sulforaphane and erucin were shown as area under peak. The sulforaphane levels were similar throughout A, B, and C ( $P>0.05$ ), while erucin were high in A comparing to B and C ( $P<0.05$ ).



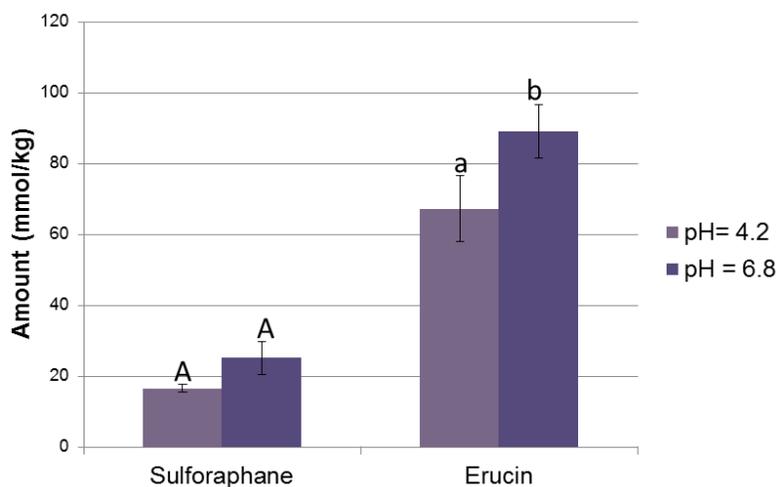
**Fig 2.** Effect of temperatures on sulforaphane and erucin formation during a 2 h hydrolysis. Different letters represent significantly different values ( $P < 0.05$ ), data are given as mean  $\pm$  SD,  $n=3$ .



**Fig 3.** Effect of time on the production of sulforaphane and erucin at 25°C. Different letters represent significantly different values ( $P < 0.05$ ), data are given as mean  $\pm$  SD,  $n=3$ .



**Fig 4.** Effect of the production of sulforaphane and erucin in water comparing to the tomato-soy-arugula seed beverage. Different letters represent significantly different values ( $P < 0.05$ ), data are given as mean  $\pm$  SD,  $n=3$ .



**Fig 5.** Effect of the pH on the production of sulforaphane and erucin in the tomato-soy beverage. Different letters represent significantly different values ( $P < 0.05$ ), data are given as mean  $\pm$  SD,  $n=3$ .

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