Food Chemistry 217 (2017) 311–319

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

# Mass-based metabolomic analysis of soybean sprouts during germination

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ARTICLE INFO

*Article history:*
Received 19 August 2015
Received in revised form 6 July 2016
Accepted 27 August 2016
Available online 31 August 2016

*Keywords:*
Soybean sprouts
Germination
Metabolomics
GC–MS
LC–MS

ABSTRACT

We investigated the metabolite profile of soybean sprouts at 0, 1, 2, 3, and 4 days after germination using gas chromatography–mass spectrometry (GC–MS) and liquid chromatography–MS (LC–MS) to understand the relationship between germination and nutritional quality. Data were analyzed by partial least squares-discriminant analysis (PLS-DA), and sprout samples were separated successfully using their PLS-DA scores. Fifty-eight metabolites, including macromolecular derivatives related to energy production, amino acids, *myo*-inositol metabolites, phytosterols, antioxidants, isoflavones, and soyasaponins, contributed to the separation. Amino acids, *myo*-inositol metabolites, isoflavone aglycones, B soyasaponins, antioxidants, and phytosterols, associated with health benefits and/or taste quality, increased with germination time while isoflavone glycosides and DDMP soyasaponins decreased. Based on these metabolites, the metabolomic pathway associated with energy production in soybean sprouts is suggested. Our data suggest that sprouting is a useful processing step to improve soybean nutritional quality, and metabolomic analysis is useful in understanding nutritional change during sprouting.


## 1. Introduction

Accumulated evidence indicates that increased consumption of plant-based foods, such as vegetables, fruits, legumes, nuts, and whole grains, is associated with a reduction in the risk of a variety of chronic diseases (Foley & Kratz, 2000). In particular, many *meta*-analyses of soybean intake have shown that a high intake of soy protein and isoflavones significantly reduced the risk of cardiovascular disease as well as cancer, and helped to alleviate osteoporosis and menopause (Ma, Qin, Wang, & Katoh, 2008; Trock, Hilakivi-Clarke, & Clarke, 2006; Zhan & Ho, 2005). Despite these health benefits, anti-nutritional factors in soybean, such as proteinase inhibitors, agglutinin, and soyatoxin, have led to limited use of soybean as a meal. To destroy anti-nutritional factors or reduce their levels in soy foods, a variety of processes, including thermal treatment, fermentation, and germination, has traditionally been used (Bau, Villaume, Nicolas, & Méjean, 1997). Among these processes, sprouting, stimulated by watering in the dark, is one of the most inexpensive and effective methods for improving the nutritional quality of legumes and for decreasing the level of anti-nutritional factors, although sprouts have a high risk of microbial contamination because their cultivation conditions (warm with high humidity) are ideal for the growth of bacteria, including *Salmonella*, *Listeria*, and *E. coli* (Roever, 1998). During sprouting, polysaccharides and proteins stored in the seeds are broken down into small components to provide energy and synthesize substrates for the early stages of seed germination, resulting in an accumulation of free amino acids and soluble carbohydrates including sucrose, glucose, and *myo*-inositol (Laila & Murtaza, 2014). Moreover, an increased level of vitamins, minerals, and other phytochemicals with various health benefits are often observed during germination in various sprouts, such as wheat and alfalfa (Plaza, Ancos, & Cano, 2003).

Similar to the sprouts of species other than soybean, the macromolecules and phytochemicals in soybean sprouts have been partially investigated using conventional targeted analysis tools (Chen & Chang, 2015; Lee et al., 2011; Shi, Nam, & Ma, 2010), but changes in the whole profile of soybean sprout compounds during germination are poorly known. To complement targeted analyses, untargeted metabolomic screening of the entire range of metabolites from biological samples has been introduced using high-throughput analysis technologies, including nuclear magnetic

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http://dx.doi.org/10.1016/j.foodchem.2016.08.113
0308-8146/

resonance spectrometry (NMR) and mass spectrometry (MS) with multivariate statistical analysis (Cevallos-Cevallos, Reyes-De-Corcuera, Etxeberria, Danyluk, & Rodrick, 2009). In recent years, comparative untargeted metabolomic analyses of various sprouts, including black gram (Jom, Chanput, & Ngampongsai, 2015), broccoli (Maldini et al., 2015), mung beans (Jom, Frank, & Engel, 2011; Tang, Dong, Ren, & He, 2014), and buckwheat (Kim, Park, & Lim, 2011) under a variety of germination conditions, as well as of soy foods, such as meju (Kang et al., 2011), soy sauce (Ko, Ahn, Berg, Lee, & Hong, 2009), and cheonggukjang (Park et al., 2010), have been performed. These metabolomic studies have been very useful in understanding the relationship between processing steps like germination and fermentation and the nutritional and/or sensory quality of sprouts and soy foods. However, metabolomic analyses of soybean sprouts in relation to germination have not yet been done. Therefore, in this study, we investigated the changing metabolomic profiles of ungerminated soybean seeds (0 days) and soybean sprouts during germination (1, 2, 3, and 4 days) using MS-based untargeted metabolomics technology. We found major metabolites associated with nutritional and/or taste quality of soybean sprouts, and have proposed a metabolomic pathway related to energy production during germination.

## 2. Materials and methods

### 2.1. Cultivation and sprouting of soybeans

Soybean seeds (*Glycine max* L. 'Choseon', 15 g) were sterilized in 150 mL of 5% NaClO (200 ppm) for 15 min, soaked in tap water for 7 h, and then placed in 0.5 L plastic pots with gauze. The seeds were sprouted in the dark at room temperature with watering every 4 h. The sprouts were collected at 1, 2, 3, and 4 days after germination. After removing the bean coats, the collected sprouts were immediately frozen in liquid nitrogen and lyophilized. Ungerminated soaked soybean seeds were used as a control (0 days).

### 2.2. β-Carotene and vitamin C analysis

β-Carotene was extracted from the soybean sprouts following the procedure of Moros, Darnoko, Cheryan, Perkins, and Jerrell (2002), with modifications. Dried samples (60 mg) were extracted with 0.6 mL of ethanol containing 0.1% butylated hydroxytoluene and the sealed extract was pre-incubated at 85 °C for 5 min. After saponification by the addition of 24 μL of 80% potassium hydroxide, the cooled crude carotenoid was separated with the addition of distilled water and hexane (1:1). The hexane layer containing the crude carotenoid was completely dried with nitrogen gas, and then the residue recovered with methanol:methyl *tert-butyl* ether (MTBE) (1:1) was used as the crude carotenoid of the soybean sprouts. The crude carotenoid was injected into an HPLC system (Shimadzu, Tokyo, Japan) equipped with a $C_{30}$ YMC column (250 × 4.6 mm, 3 μm; YMC Co., Ltd, Tokyo, Japan) to analyze the β-carotene. The mobile phase consisted of methanol:MTBE:water (81:15:4, solvent A) and methanol:MTBE (9:91, solvent B) with a gradient elution over 24 min at a flow rate of 1.5 mL/min. The eluent was detected with a Shimadzu SPD-M20A photodiode array detector at 450 nm. For analysis of vitamin C, dried samples (75 mg) were extracted with 1.5 mL of 5% metaphosphoric acid (Gardner, White, McPhail, & Duthie, 2000). The extracts were defatted with chloroform and injected into an HPLC system equipped with a $C_{18}$ Shim–pack GIS–DOS (250 mm × 4.6 mm, 5 μm; Shimadzu). The mobile phase consisted of 25 mM $KH_2PO_4$ with an isocratic elution over 10 min at a flow rate of 0.8 mL/min and the eluent was detected at 254 nm. Authentic β-carotene and vitamin C were used to evaluate quality and quantity in the soybean sprouts.

### 2.3. Determination of total content of phenolic compounds

The total amount of phenolic compounds in soybean sprouts extracted with 50% methanol was determined using Folin-Ciocalteu reagent (Sigma-Aldrich, St. Louis, MO, USA) by the method of Singleton and Rossi (1965). A reaction mixture of 50 μL of the extract, 450 μL of distilled water, 250 μL of 2 N Folin-Ciocalteu reagent, and 1.25 mL of 20% $Na_2CO_3$ was incubated at 25 °C for 20 min. After centrifugation, the absorbance of the supernatant was measured at 735 nm. The standard curve was prepared using gallic acid (GA) and the phenolic content was expressed in terms of GA equivalent (mg of GAE/g of sample dry weight).

### 2.4. Metabolite extraction and derivatization for GC/MS analysis

For GC/MS analysis of soybean sprout metabolites, samples were extracted with 50% MeOH and then chloroform was added to the extract. The supernatant (methanol/water layer) was dried using a vacuum CentriVap concentrator (Labconco, Kansas City, MO, USA) at 40 °C. The dried samples were dissolved in 2% methoxyamine hydrochloride in pyridine, with dicyclohexyl phthalate as an internal standard, and were incubated at 37 °C for 90 min. After the methoxylation, samples were derivatized with 100 μL *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and 1% trimethylchlorosilane (TMSC) at 70 °C for 30 min. The derivatized samples were analyzed by GC/MS (Fiehn, Kopka, Trethewey, & Willmitzer, 2000).

For phytosterol analysis, 100 mg of dried sample was extracted with 2 mL of 0.5 M KOH/MeOH at 90 °C for 15 min. The extract was mixed with 1 mL of boron trifluoride (BF3) in methanol and incubated at the same temperature for 2 min. After cooling, 2.5 mL of saturated sodium chloride solution and 2 mL of hexane were added to the reaction mixture. After centrifugation, 500 μL of the hexane layer was derivatized with 150 μL of BSTFA at 70 °C for 20 min, with 20 μL of 5α-cholestane as an internal standard (Hwang, Wang, & Choong, 2003).

### 2.5. GC/MS analysis

The derivatized 50% methanolic and phytosterol extracts were analyzed using a Shimadzu GC-2010 plus (Tokyo, Japan) equipped with a DB-5 ms capillary column (30 m × 0.25 mm, 0.25 μm, Agilent J&W, Santa Clara, CA, USA) and an Rtx-5 ms capillary column (30 m × 0.25 mm, 0.25 μm, Restek Co., Bellefonte, PA, USA), respectively. One microliter each of methanolic and phytosterol extract was injected into the capillary columns with split ratios of 1:50 and 1:5, respectively. Helium was used as a carrier gas at a flow rate of 1 mL/min and the injection temperature was set at 250 °C. The oven temperature was programmed from 70 °C to 320 °C at a rate of 6 °C/min with initial and final holding times of 2 min and 8 min, and from 80 °C to 320 °C at a rate of 12 °C/min with the same hold times, for analysis of the 50% methanol and phytosterol extracts, respectively. The GC column effluent was detected with a Shimadzu GCMS-TQ 8030 mass spectrometer (Tokyo, Japan) in electron ionization (EI) mode (70 eV). The temperatures of source and interface were 230 °C and 280 °C, respectively. The MS spectra were monitored in the full scan mode from *m/z* 45–550 with a scan event time of 0.3 s and a scan speed of 2000 μ/s.