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Vol. 7(17), pp. 1129-1137, 3 May, 2013
DOI: 10.5897/JMPR11.1507
ISSN 1996-0875 ©2013 Academic Journals
http://www.academicjournals.org/JMPR

Journal of Medicinal Plants Research

Full Length Research Paper

Far infrared assisted conversion of isoflavones and its effect on total phenolics and antioxidant activity in black soybean seed

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Accepted 3 January, 2012

Aglycone isoflavones are more potent in their biological activities than their corresponding glycosides. The objective of this research was to explore the suitable temperature and time to obtain optimum conversion of daidzein and genistein using far infrared irradiation (FIR) as a thermal source on black soybean. The changes were monitored and quantified by high performance liquid chromatography (HPLC). The alteration in total phenolics (TP) and antioxidant activity due to FIR in different temperature and time was also evaluated. The increase in temperature with increasing exposure time in FIR caused gradual increase in diadzein and genistein production. The average maximum increase in aglycone isoflavone (sum of diadzein and genistein) content was achieved in 30 min of exposure with 341 mg/100 g dw at 196±6°C which were about 9.89 and 5.08 fold higher than those of the control for genistein and diadzein, respectively. The TP contents also altered due to the increase in temperature and exposure time and reached a plateau (1385.31 mgTAE/100 g dw) after about 30 min of irradiation at 196°C which was 2.87 fold higher than the control. In antioxidant assay, the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical inhibition was increased with temperature and time. However, the metal chelating property decreased with increasing temperature. Overall, the FIR can be used as a convenient tool for chemical conversion of isoflavone glycosides in soybean.

Key words: Antioxidant, conversion, isoflavone, far infrared irradiation, high performance liquid chromatography, soybean.

INTRODUCTION

Soybeans (*Glycine max* M.) are rich in phytochemicals, especially isoflavones. Medical studies have shown that the consumption of soybeans decreases the risk of various diseases like cancer (Anthony et al., 1996); osteoporosis (Arjmandi et al., 1998); renal diseases (Fico et al., 2000) and coronary heart disease (Lucas et al., 2001). There are also reports to improve menopause symptoms (Potter et al., 1998; Ishimi et al., 2002); and antidiadetic effects (Liu et al., 2006) by soybean isoflavones.

In soybeans, three different types of isoflavones viz diadzin, genistin and glycitin are present in abundant amounts in the form of their glycosides and only in a small percentage as the principal bioactive aglycones (Wang et al., 1998). Several reports have confirmed that the isoflavones in the form of aglycones display a higher biological activities than isoflavones in the form of glucosides (Onozawa et al., 1998; Peterson and Barnes,

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1991; Piskula et al., 1999). Among the different isoflavones present, genistein and daidzein are more potent in the biological activities than other isoflavones (Lee et al., 2005; Markham et al., 1978). These mentioned aglycone isoflavones are responsible to provide protection against oxidative modification of low-density lipoprotein (LDL) particles in human volunteers (Tikkanen et al., 1998). In addition, genistein and daidzein also possess strong antioxidant potency (Record et al., 1995; Lee et al., 2005). Furthermore, Genistein has higher antiproliferative activity in the growth of human breast carcinoma and prostate cancer cells than genistin (Onozawa et al., 1998; Peterson and Barnes, 1991; Jun et al., 2004).

Previous research has indicated that isoflavones were not destroyed under normal thermal processing conditions but rather were subjected to interconversions between the different forms (Murphy et al., 2002). Although, several attempts have been made to study stability and conversions of isoflavones, there is not much available literature about the far infrared (FIR) thermal effect on raw soybean and the chemical modification of the isoflavones caused by FIR. Nowadays, several reports have shown that FIR drying method is more advantageous than conventional oven drying method (Eom et al., 2009; Lee et al., 2003; Kim et al., 2006). The high penetration power of FIR helps to stimulate exudation of chemical components without destroying the plant cells (Niwa et al., 1988; Eom et al., 2009) and thereby causes the alteration of biological activity. Since genistein and diadzein (aglycones) are believed to have better health benefits, their safe and easy production without using expensive enzymes or hazardous chemicals is important from a health point of view. In this research, our main objective was to explore the suitable temperature and time to obtain the maximum conversion of aglycone isoflavones (daidzein, genistein) using FIR as a thermal source on black soybean. Furthermore, we also monitored the alteration caused by FIR on total phenolics content and biological (antioxidant) activities.

MATERIALS AND METHODS

Chemicals

Organic solvents (methanol, acetonotrile, ethanol) used for the extraction of plant materials and detection in HPLC were purchased as analytical grade from Merck KGaA Darmstadt, Germany. Tannic acid, and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma Chemical Co. (St. Louis, Mo) and Folin-Ciocalteu reagent was from Wako Pure Chemicals, Japan. The pure compounds like diadzin, genistin, daidzein and genistein were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals and reagents used were of the highest commercially available purity.

Plant materials and extraction procedures

Black soybean seeds were bought from local market (Chuncheon City, Kangwon Do, Korea) and made to powder (10 powder using mixture grinder). Two grams of powdered sample in a glass petri disc (10 × 20 mm) were exposed to far infrared (Korea Energy Co., Seoul, Korea) drier emitting wavelengths of 3 to 1000 µm. The energy flow of the FIR drier was set up at 50, 60 and 70%, which showed the average temperature of 143, 166 and 196°C with ±6°C fluctuation respectively. The sample exposed time was set up at the interval of 15 min (15 to 60 min) for 143 and 166°C. However, there could be burning effect in higher temperature; therefore, 10 min interval (10 to 40 min) was set up for 196°C. After treatment, the samples were suspended in 200 ml of 70% ethanol (v/v) and kept overnight in a shaker at room temperature. The extracts were filtered and concentrated with a vacuum rotary evaporator (EYLA N-1000, Tokyo, Japan) in a 40°C water bath. Dried samples were weighed and kept at 4°C for further analysis.

Estimation of total phenolic (TC) content

The total phenolic (TP) content was determined by the Folin-Ciocalteu assay (Eom et al., 2008). In brief, a sample aliquot of 1 ml of extract (1 mg/ml) was added to a test tube containing 200 µl of phenol reagent (1 M). The volume was increased by adding 1.8 ml of distilled deionized water and the solution was allowed to stand for 3 min for reaction after vortex. Further, 400 µl of Na_2CO_3 (10% in water, v/v) was added and the final volume (4 ml) was adjusted by adding 0.6 ml of deionized water. A reagent blank was prepared using deionized water. The absorbance was measured by spectrophotometer (U-2001, Hitachi, Japan) at 725 nm after incubation for 1 h at room temperature. The TP was calculated from a calibration curve (R^2=0.9914) using tannic acid as a standard and the obtained average value was expressed as tannic acid equivalents (TAE) in mg/100 g dry weight of the sample.

DPPH free radical scavenging activity

The antioxidant activity of treated sample was determined on the basis of the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical according to the method described by Braca et al. (2003) with slight modifications. Briefly, 1 ml of each of the extracts at the concentration of 2 mg/ml was added to 3 ml (0.15 mM methanol solution) of DPPH. The mixtures were shaken vigorously and left to stand at room temperature in the dark for 30 min. The absorbance was measured at 517 nm in a spectrophotometer (Hitachi U-2001, Japan) and the percent inhibition activities of the extracts were calculated against a blank.

Radical scavenging activity (%) = $(A_0 – A_1)/A_0 \times 100$

Where, A_0 and A_1 was the absorbance of control and test sample respectively.

Ferrous ion chelating ability

The chelation of Fe^{2+} by the extracts was measured according to the method of Dinis et al. (1994) with minor modification. Briefly, 0.5 ml of the sample at the concentration of 0.5 mg/ml of extract was incubated with 0.1 ml $FeCl_2$. $4H_2O$ (1 mM). The reaction was initiated by the addition of 0.2 ml ferrozine (5 mM) and vortexed and kept for 10 min. The absorbance of the mixtures was measured against the blank in a spectrophotometer (ELX800TM, BioTek, USA) at 562 nm after the addition of 3.2 ml 80% ethanol (total volume 4 ml). The test solution without sample served as the negative control. The metal chelation (%) was calculated using the following formula:

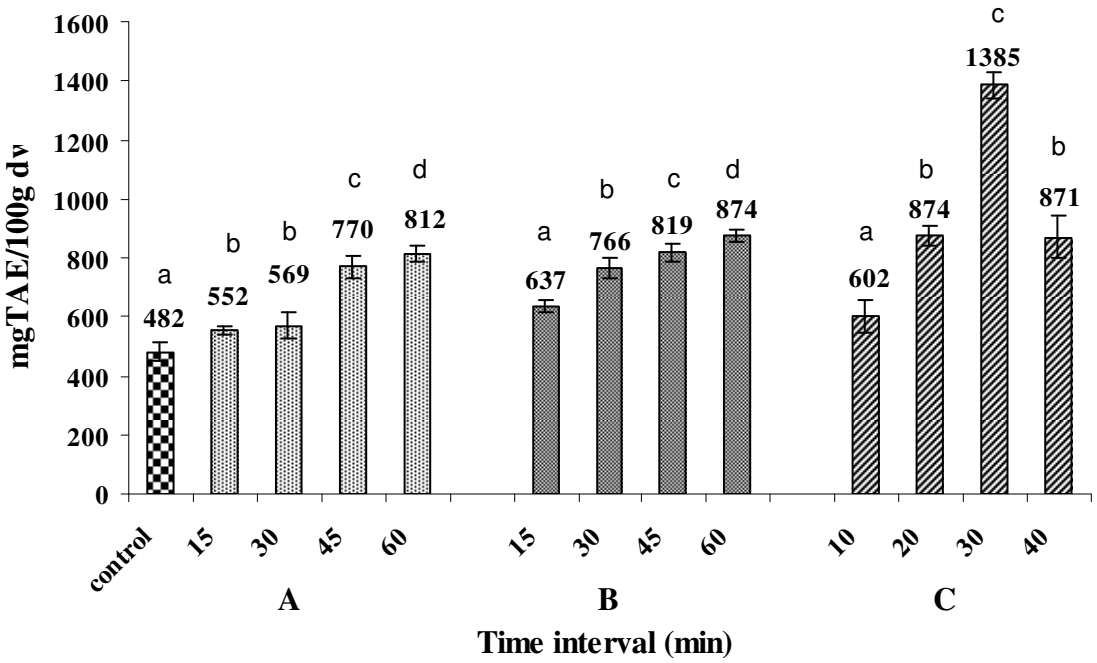


Figure 1. Total polyphenol content of FIR treated and untreated (control) soybean sample expressed in tannic acid equivalent (TAE) in mg/100g dw. All values are expressed in average (n=3). The letter A, B and C represent temperature at 143, 166 and 196°C (±6°C fluctuation) in different time intervals respectively. Mean values within a row not sharing a common superscript letters (a, b, c, d) were significantly different ($p \leq 0.05$).

Chelation (%) = $(A_0 - A_1)/ A_0 \times 100$

Where, A_0 was the absorbance of the negative control and A_1 was the absorbance with the test samples.

HPLC quantification of isoflavones in FIR treated and untreated soybean

To quantify the isoflavones content in the FIR treated and untreated extracts, high-performance liquid chromatography (HPLC) system (CBM-20A; Shimadzu Co, Ltd., Kyoto, Japan) with 2 gradient pump systems (LC-20AT; Shimadzu, Japan), an auto sample injector (SIL-20A; Shimadzu), a UV-detector (SPD-10A; Shimadzu) and a column oven (CTO-20A; Shimadzu) were used for analysis. The separation was performed on a C_{18} column (Synergi 4µ MAX-RY, 150 × 4.6 mm, 4 micron Phenomenex. Inc., Torrance, CA, USA). Flow rate of mobile phage solution was 1.0 ml/min, and detection was at 280 nm. 10 µl of each sample was injected. The compounds were identified in solvents by matching their retention times and spectra with that of the standards (daidzin, genistin, daidzein, genistein) and the data were calculated on the basis of the peak area obtained. The mean data obtained was expressed in mg/100 g dw.

Statistical analysis

All data were expressed as mean value ± standard deviation (SD) of the number of experiments (n=3). Data analysis was performed using SPSS 16 (Institute, Cary, NC, USA); individual comparison was made using Duncan's multiple-range test, which was used to determine the difference between the means.

RESULTS

Effect of FIR on total phenolics (TP) content in soybean

The plant phenolics constitute one of the major groups of compounds acting as antioxidants or free radical terminators (Miliauskas et al., 2004). Therefore, the content of TP in the FIR treated and untreated (control) was determined in the soybean extracts (Figure 1). The data revealed that the increase in temperature and exposure time caused gradual increase in TP content in FIR treated soybean. Compared with the control (482 mgTAE/100 g dw), the increase in TP was 812 mgTAE/100 g dw which was nearly 1.68-fold higher after exposure at 143°C for 60 min. Likewise, further increase in temperature to 166°C caused 1.81-fold increase in TP. The maximum increase was obtained after exposure of sample for 30 min at 196°C with 1385.31 mgTAE/100 g dw. This increment was about 2.87-fold higher than that of the control.

Changes in DPPH free radical scavenging activity

The effect of phenolic compounds on DPPH radical scavenging is thought to be due to their hydrogen donating ability. It is reported that the decrease in the absorbance of DPPH radical caused by phenolic compound is due to

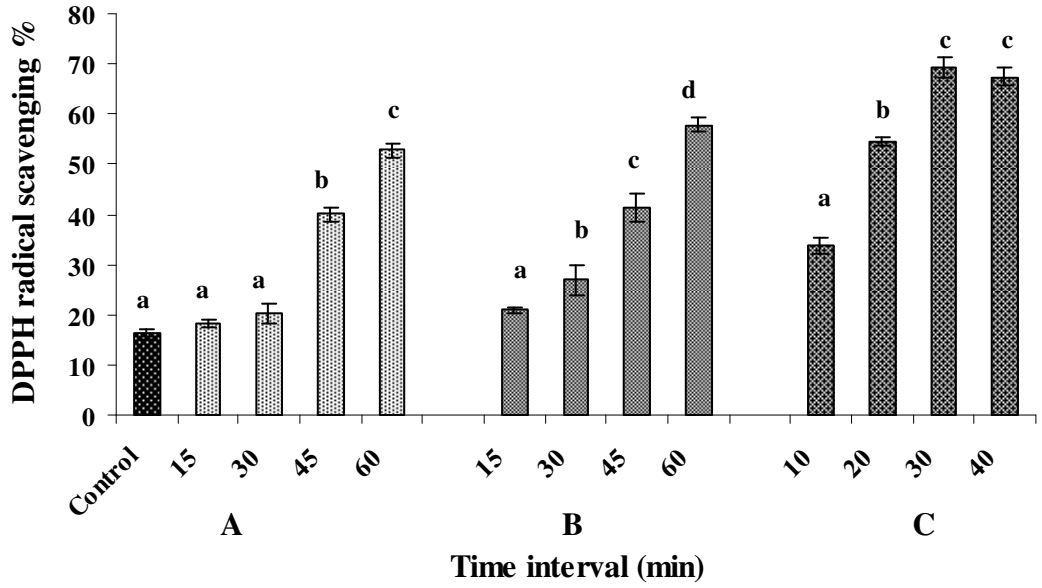


Figure 2. DPPH free radical scavenging activity of FIR treated and untreated soybean sample expressed in percentage. The samples were used at the concentration of 2 mg/ml for comparison. Absorbance was taken at 517 nm. All values are expressed in average (n=3). The letter A, B and C represent temperature at 143, 166 and 196°C in different time intervals respectively. Mean values within a row not sharing a common superscript letters (a, b, c, d) were significantly different (p≤0.05).

the reaction between antioxidant molecules and radicals, resulting in the scavenging of the radical by hydrogen donation and is visualized as a discoloration from purple to yellow (Meir et al., 1995). According to our research, FIR affects the free radical scavenging activity of the soybean (Figure 2). The samples exposed to FIR showed a gradual increase in the DPPH free radical scavenging activity in a temperature and time dependent manner. At the temperature of 143 and 166°C, the inhibition percent was about 52.79 and 57.91 respectively after 60 min of exposure. Further exposure of sample at 196°C in different time intervals showed higher scavenging activity, gaining a maximum value after 30 min with 69.18% inhibition, which was 4.28-fold higher than the control (16.16%) at the concentration of 2 mg/ml. The exposure of the sample for a longer time (40 min) at a higher temperature of 196°C caused a decline in the inhibition percent (67.39%) due to the over burning of the sample that probably destroyed the active antioxidant components.

Correlation between free radical scavenging activity and the phenolic contents in FIR treated soybean

The correlation between the free radical scavenging activity and phenolic content due to the heating effect in soybean is shown in Figure 3. The correlation coefficient between the data of TP and DPPH scavenging percent was 0.703, conforming that increase in phenolic compound was likely to contribute to the radical scavenging activity of the soybean extracts. This correlation between the free radical scavenging activity and increased TP was probably due to the release in aglycone compounds or maillard products that can react as an electron donor or transfer a hydrogen atom to the DPPH radical and thus increasing the antioxidant properties (Ruanma et al., 2010).

Ferrous ion chelating ability

Metal chelating agents that form bonds with a metal are effective as secondary antioxidants because they reduce the redox potential and thereby stabilize the oxidized form of the metal ion (Keowmaneechai and McClements, 2006). In this assay, the metal chelating property of soybean was altered due to temperature and exposure time (Figure 4). The result revealed that the exposure of sample at low temperature (143°C) enhanced the metal chelation property in soybean. The maximum chelating property was observed at 143°C with the inhibition of 74.73% at 30 min of exposure. Likewise, short time (10 to 15 min exposure at 166 and 196°C) exposure also enhanced the chelating property, suggesting the presence of polyphenols that has potent iron chelating capacity in soybean. However, the over exposure of sample decreased the property. Therefore, this study showed that FIR treatment in higher temperature for long time decreased the metal chelating property in soybean. However, exposure in a low temperature (143°C) or with short time (10 min) at a higher temperature (at 166 or 196°C) can enhance iron binding capacity.

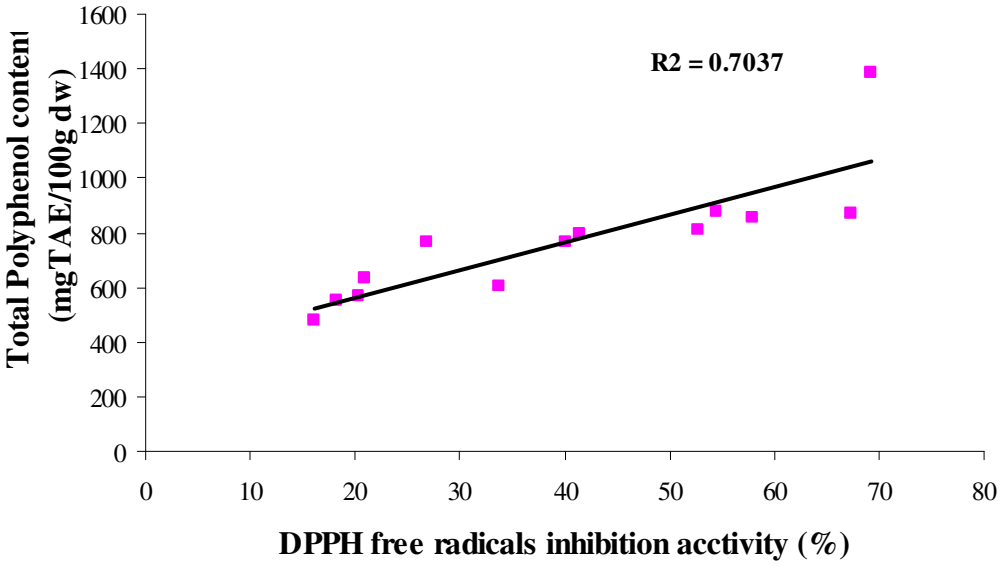


Figure 3. Linear correlation plot of the total phenolic (TP) content and DPPH free radical scavenging activity (%).

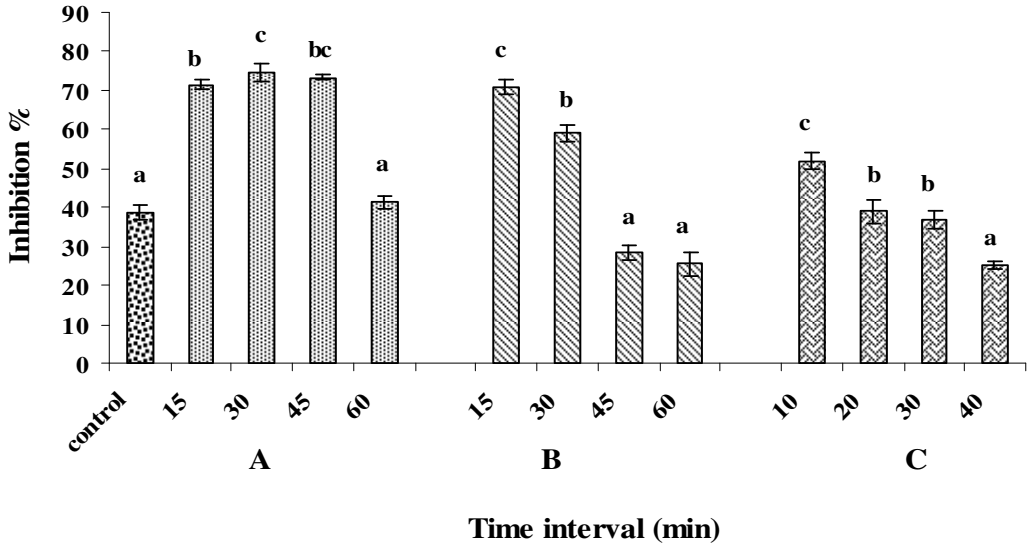


Figure 4. Metal chelating property of FIR treated and untreated (control) soybean sample expressed in percentage. The samples were compared at the concentration of 0.5 mg/ml. Absorbance was taken at 562 nm. All values are expressed in average (n=3). The letter A, B and C represent temperature at 143, 166 and 196°C in different time intervals respectively. Mean values within a row not sharing a common superscript letters (a, b, c, d) were significantly different (p≤0.05).

HPLC quantification of isoflavone in FIR treated and untreated soybean sample

Table 1 shows the effect of FIR on soybean in different temperature and time intervals and, Figure 5 shows the pattern of chromatograms of different isoflavones formed in different time intervals at the temperature of 196°C with maximum aglycone production. According to the HPLC profile, the aglycone isoflavones increased gradually with the increase in exposure time. The gradual increment was observed from 23.631 to 80.047 mg/100 g and 21.378 to 116.56 mg/100 g dw for daidzein and genistein respectively from 15 to 60 min at 143°C. Likewise, these compounds further increased to 89.89 and 131.442 mg/100 g dw respectively after exposure of 1 h at about 169°C. The maximum increment was observed at 196°C after 30 and 40 min of exposure, accounting for 204.094 and 144.97 mg/100 g dw for daidzein and genistein, respectively. However, considering the total amount (sum of daidzein and genistein was 324.06 mg/100 g dw) content,

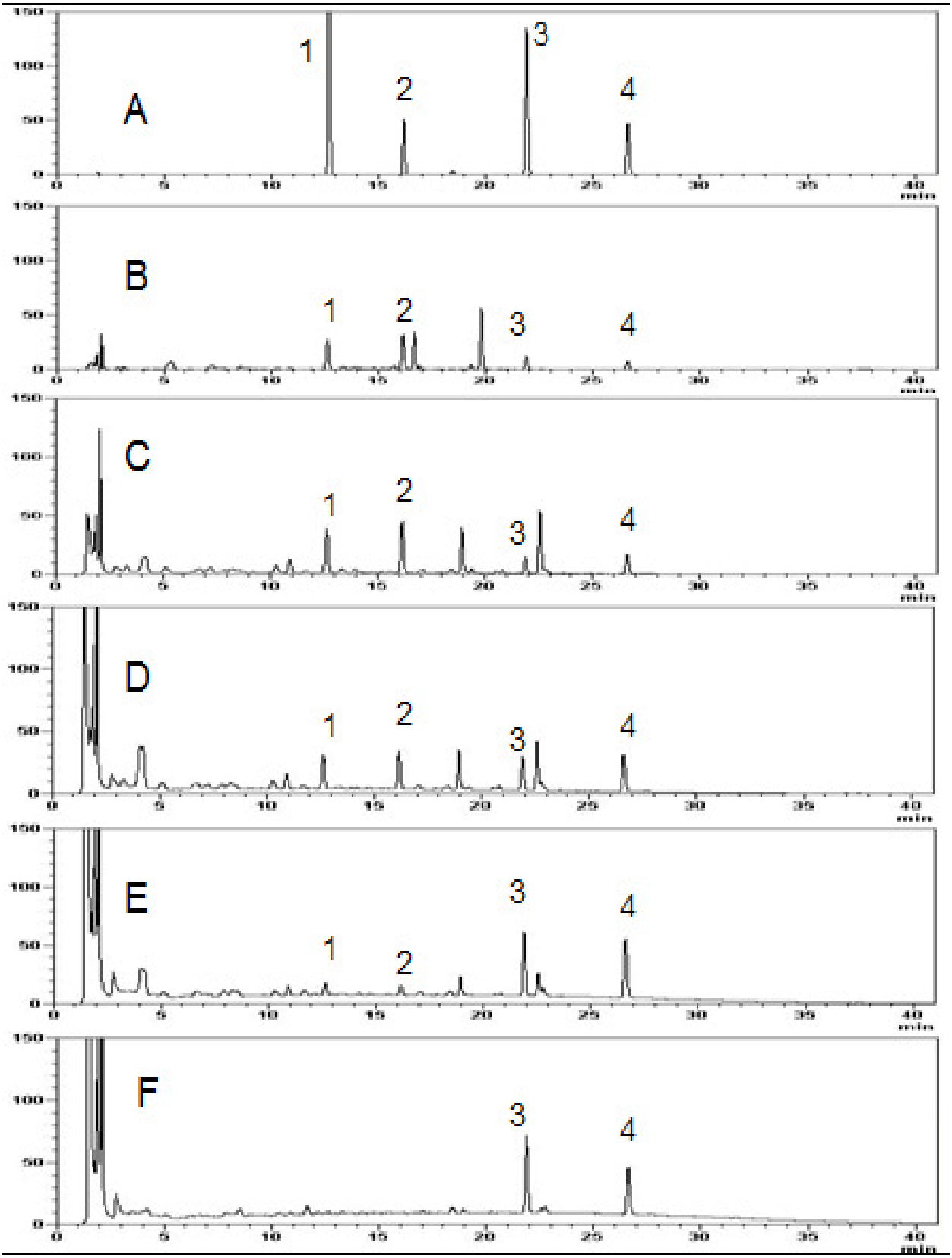


Figure 5. HPLC chromatogram of daidzein, genistin, daidzein and genistein content in FIR treated and untreated soybean seed sample. 'A' denotes the peaks of the external standard used. 1, 2, 3 and 4 represents diadzin, genistin, daidzein and genistein respectively. 'B' represents untreated (control) and C, D, E and F represent FIR treated samples at 196°C for 10, 20, 30 and 40 min respectively.

Table 1. HPLC quantification of daidzin, genistin, daidzein and genistein content in FIR treated at 143, 166 and 196°C (±6°C fluctuation) in different time intervals and untreated (control) soybean seed sample. The average values were expressed in mg/100 g dw. The letters D, G, De and Ge represents daidzin, genistin, daidzein and genistein respectively.

Temperature (°C)	Sample time interval (min)	Daidzin (D)	Genistin (G)	Daidzein (De)	Genistein (Ge)	Total (De+Ge)	Total (D+G+De+Ge)
	Control	78.48^{a}	76.50^{a}	23.58^{a}	20.63^{a}	44.21^{a}	199.20
143	15	89.15^{b}	118.35^{c}	23.63^{a}	21.38^{a}	42.01^{a}	252.50
	30	89.53^{b}	101.26^{b}	49.59^{b}	38.69^{b}	68.37^{b}	279.06
	45	100.60^{c}	96.84^{b}	53.11^{b}	72.12^{c}	125.23^{c}	322.67
	60	88.22^{b}	73.69^{a}	80.05^{c}	116.56^{d}	196.61^{d}	358.51
166	15	87.57^{ab}	101.64^{c}	27.63^{a}	48.89^{a}	76.53^{a}	265.73
	30	88.13^{ab}	99.04^{c}	75.81^{b}	113.52^{b}	189.33^{b}	376.50
	45	93.49^{c}	86.43^{b}	77.27^{b}	124.30^{b}	201.57^{c}	381.49
	60	81.86^{a}	72.61^{a}	89.59^{c}	131.44^{c}	221.03^{d}	375.50
196	10	112.05^{d}	106.43^{d}	25.29^{a}	41.93^{a}	67.22^{a}	285.71
	20	106.62^{c}	93.58^{c}	55.78^{b}	145.77^{b}	201.56^{b}	401.76
	30	40.65^{b}	28.01^{b}	119.97^{c}	204.09^{d}	324.06^{d}	392.71
	40	14.19^{a}	11.92^{a}	144.97^{d}	171.10^{c}	316.07^{c}	342.18

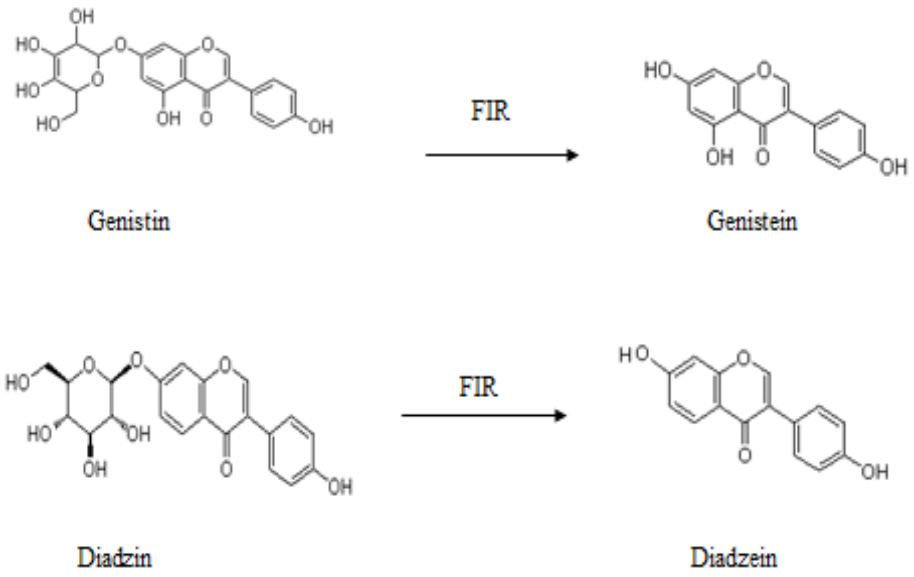
Mean values within a row not sharing a common superscript letters (a, b, c, d) were significantly different ($p \leq 0.05$).

content, 30 min exposure at around 196°C was the best condition for the production of aglycone isoflavones. Over exposure (more than 40 min) in higher temperature (196°C) caused gradual decline in total isoflavones content.

DISCUSSION

Thermal processing causes chemical, physical and biological changes in plants and plant products (Hoyen and Kvale, 1977). In soybeans, the heat treatment can alter the form of isoflavones (Mathias et al., 2006). In our research, the use of FIR as a thermal source caused maximum increase in TP content in a temperature and time dependent manner in black soybean seed. The maximum increment was observed in 20 min of exposure at 196°C which was about 2.76 fold higher for TP content compared to the control. The data also showed a gradual increase in DPPH free radical scavenging activity in a time and temperature dependent manner (Figure 2) and had a good correlation (0.703) with TP content. This increased free radical activity of soybean could be contributed to the synergistic effect of all isoflavones and other phenolic compounds like caffic acid, chlorogenic acids or tocoferol in the soybean. In previous report, Kumar et al. (2009) mentioned that these later compounds would increase during heat processing and contribute higher radical scavenging activity in soybean than its isoflavones. In previous study, there are several reports of increase in antioxidant property due to thermal effect in different plants like onion (Woo et al., 2007), grape (Eom et al., 2009), citrus peel (Xu et al., 2007), etc. In case of metal chelating property, the effect was higher in lower temperature (143°C) or in short time (10 to 15 min) exposure at about 166 and 196°C. This effect could be credited to the higher genistin accumulation in lower temperature or short time exposure in higher temperature (Table 1), because the genistin can be a better metal chelator than other isoflavones (Lee et al., 2005).

In this research, the HPLC data revealed that the FIR affected the isoflavones of soybean in different extents. During the experiment, the slight increase in isoflavone glycosides (genistin and daidzin) occurred in short time exposure in the given temperature (Table 1). However, long time exposure at about 143 and 166°C or after 20 min of exposure at 196°C gradually decreased the contents. In contrast to aglycone isoflavones, genistein and daidzein formation from their corresponding glycosides occurred in a temperature and time dependent manner in FIR treated soybean (Figure 5). In overall comparison of total amount of aglycone isoflavone (sum of daidzein and genistein was 324.06 mg/100g dw) content, 30 min exposure at about 196°C was the best temperature for the maximum conversion to aglycone isoflavones (Table 1). This conversion could be due to the instability of isoflavone glycosides or malonylglycosides and acetylglycosides in soybean (Murphy et al., 2002). FIR has higher penetration power (up to 2 cm), therefore, such type of increase in phenolics and transformation is possible where high molecular phenolics converts to low molecular compounds by breaking of phenolics covalent



Scheme 1. Conversion of glycoside isoflavones (genistin and diadzin) to aglycone isoflavones (genistein and daidzein) using far infrared irradiation (FIR).

bonds by FIR treatments (Scheme 1) (Niwa et al., 1988; Eom et al., 2009). Although, the proper mechanism of deglycosilation by FIR is unknown, the breakage of isoflavones glucosidal bond could be due to the inter-molecular reactions and vibration caused by far infrared (electromagnetic wave) rays (Iiyasov and Krasnikov, 1990). The data also revealed that over exposure of the sample in higher temperature (above 40 min at 196°C) in FIR caused a decrease in isoflavone production due to thermal degradation of the phenolics in the soybean. In previous studies, there were also reports of degradation of isoflavones during different thermal processes in soybean, tofu, and soymilk (Grun et al., 2001; Murphy et al., 2002).

Conclusion

These results highlighted that FIR irradiation enhanced the aglycone isoflavones production (genistein and diadzein) in a time and temperature dependent manner in black soybean seed with the maximum production at about 196°C for 30 min. And also, FIR treatment increased the TP content thereby altering the antioxidant (free radical scavenging, metal chelating capacity) activity. Overall, this result presents invaluable information to soy food producers aiming towards improving the nutritional value of their product through enhancing isoflavones by using FIR as a thermal source.

ACKNOWLEDGEMENTS

This study was partially supported by the Research Institute of Bioscience and Biotechnology, Kangwon National University, South Korea.

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