Thesis for the Degree of Master of Engineering

Characterization and Hydrolytic Kinetics Modeling of Okara Isoflavones Recovered from Subcritical Water Process

by

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아임계 수를 이용하여 추출한 콩비지 이소플라본의 특성 및 가수분해 속도 모델링

Advisor: Prof. Byung-Soo Chun

by

David Nkurunziza

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Characterization and Hydrolytic Kinetics Modeling of Okara Isoflavones Recovered from Subcritical Water Process

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Abstract

This study investigated the potential of using subcritical water extraction process (SWE) for the recovery of valuable bioactive materials from soybean by-products (okara) that are produced after soymilk and tofu processing. In the first study okara was exposed to subcritical water extraction with the intention of recovering isoflavones with minimal degradation products. Response Surface Methodology (RSM) of the extraction variables indicated optimized conditions would be T = 146.23°C, P = 39.8 bar, and α= 20 mg (solid)/mL (extractant). Mathematical models of the rates of reactions for each genistein-based and daidzein-based compounds were solved as a set of simultaneous equations leading to rate constants and time-dependent concentration profiles for each compound. These kinetics analyses suggested an optimum extraction time at the SRM-optimized conditions would be 213.5 ±8.7 min. The model will provide people involved in soybean industry and academics with a deep knowledge of managing conversions and degradations reaction kinetics of isoflavones during heat processing of soybean and soybean by-products.

The second part of this study focuses on the Valorization of underutilized Okara resources to create economic benefits by processing it into a product with a high content of isoflavone aglycones, phenolic content and antioxidant activities using subcritical water. Extraction conditions for key variables were kept constant from the optimum

conditions obtained in the previous study (temperature: 146±5°C, pressure: 4MPa, mixing ratio: 20mg/mL) for the optimization of isoflavones recovery. The reaction time was considered as a key parameter to increase the content of more bioavailable unconjugated isoflavone aglycones and the total phenolic contents of the hydrolysates. The initial reaction time of 5 min was extended up to 275 min in increments of 30 min. A significant increase in 2 isoflavone aglycones genistein and daidzein up to 6.5 and 9 fold was observed respectively. The results confirm the time dependent conversion of conjugated isoflavones to aglycones, On the other hand total phenolic content (TPC) increased up to 68 % the initial content after 245 min. Surprisingly the antioxidant activities of the hydrolysates display a strong correlation between the TPC, isoflavone aglycones and antioxidant activities (ABTS, DPPH and FRAP). The Pearson correlation and Principal Component Analysis (PCA) were used to analyze the relationship between the variables. These findings suggested that the increase in reaction time improved the antioxidant activities, isoflavone aglycones and total phenolics of hydrolysates.

CHAPTER 1

General Introduction

1.1. Soybean processing, isoflavones and health benefits

Soybean (*glycine max*) is the most extensively grown and consumed legume in the world, rich in proteins and edible oil, and a variety of micronutrients and phytochemicals [1]. The processing of soymilk and tofu leaves out a massive amount of byproducts named Okara. These byproducts are produced in high quantities, at every kilogram of soybeans processed into soymilk 1.1 kg of byproducts is generated [2]. This byproduct is underutilized or regarded as waste because of difficulties in handling mainly from its high moisture content (70-80%) leading to its disposal.

The environmental problems from disposing these highly produced byproducts can be huge since its putrefaction may result in elevated carbon dioxide production. However it has a huge potential to be used as inexpensive source of functional ingredients. It is rich in fiber, fat, proteins, vitamins, trace elements and polyphenols. The most known polyphenols are isoflavones and other phenolic acids [3]. These compounds exhibit the antioxidant activities in vitro and shows the potential of preventing the estrogen receptor positive caused breast cancer [4]. Isoflavones are also linked with the prevention or delay of menopausal symptoms in women. They are regarded as phytoestrogenic compounds because of their similarities with mammalian estrogen hormone 17β-Estradiol which have attracted more research about their functional benefits for human being, therefore considering Okara as an inexpensive

resources of isoflavones, other phenolics and antioxidants would be of huge benefit economically and environmentally.

Isoflavones from soybean are classified into four groups depending on their chemical conformation and the presence of conjugated groups **(Table1.1).** Three isoflavones, aglycones (genistein (GE), daidzein (DE), and glycitein) are non-conjugated native forms whereas the β-glucosides (genistin (GI), daidzin (DI) and glycitin) are conjugated forms containing a glucose unit. Malonylglucosides, consisting of 6"-o-malonylgenistin (MG), 6"-o-malonyldaidzin (MD), and 6"-o-malonylglycitin and the acetylglucosides 6"-o-acetylgenistin (AG), 6"-o-acetyldaidzin (AD), and6"-o-acetylglycitin are results of heat processing, converting the malonyl group through esterification [5]. Compounds MG, GI, and DI account for 83%–95% of total isoflavones in soybean [6].

Table 1.1. Isoflavones profiles in soybean and soybean derived products

Compounds	**Isoflavones**
Aglycones	Daidzein Genistein Glycitein
B-Glucosides	Daidzin Genistin Glycitin
Acetylglucosides	Acetyldaidzin Acetylgenistin Acetylglycitin
Malonylglucosides	Malonyldaidzin Malonylgenistin Malonylglycitin

The biological activities of isoflavones have been correlated with their chemical structures, and the number of hydroxyl groups in their nucleus showed a strong relationship with the extent of their antioxidative capacity while the glucosidation of isoflavones lower their antioxidative activities significantly [7]. The antioxidant activities of fermented soybean products showed that the increase in isoflavone aglycones through cleavage of conjugated groups increased the antioxidant activities of the resulting products [8]. The theoretical investigation of antioxidant activity mechanisms of non-conjugated aglycones (daidzein, genistein and glycitein) gives an insight about the relationship between increased antioxidant activities when aglycones increases, these compounds form stable complexes with free radicals that causes oxidative stresses [9]. Although aglycones are more easily absorbed by humans than their conjugated counterparts and exhibit biological activities they exist at low concentrations in unprocessed products (below 5%, compared to over 95 % for conjugated isoflavones) [10, 11].

1.2. Isoflavones profiles in soybean and by-products

Isoflavones chemical structures are affected by the processing techniques applied **(Figure 1.1).** Fermentation and heat processing of soybean and soybean derived products yields a high concentration of non-conjugated isoflavone aglycones from the hydrolysis of conjugated isoflavone glycosides. However heat processing techniques result in quick degradation of isoflavones and care should be taken during this kind of processes to yield a significant amount of isoflavones. Several processing methods have been employed to study the chemical conversions and degradations of isoflavones and the results obtained have led to the development of different kinetics models that

describe the conversions and degradations of soybean isoflavones in different food processing systems. Chien *et al*. developed a model for the degradation and conversion of isoflavones during both moist and dry heating conditions (100–200°C) [12]. Niamnuy *et al*. investigated the drying kinetics of soybean during gas-fired, infrared heating via hot air using different temperatures [13]. Also, using a circulating-hot-air, oven drying of okara, Muliterno *et al*. revealed the conversion of isoflavone glucosides to aglycones and a reduction of isoflavones content at 70°C, with the drying kinetics best fitted with Fick and Page diffusion models [14].

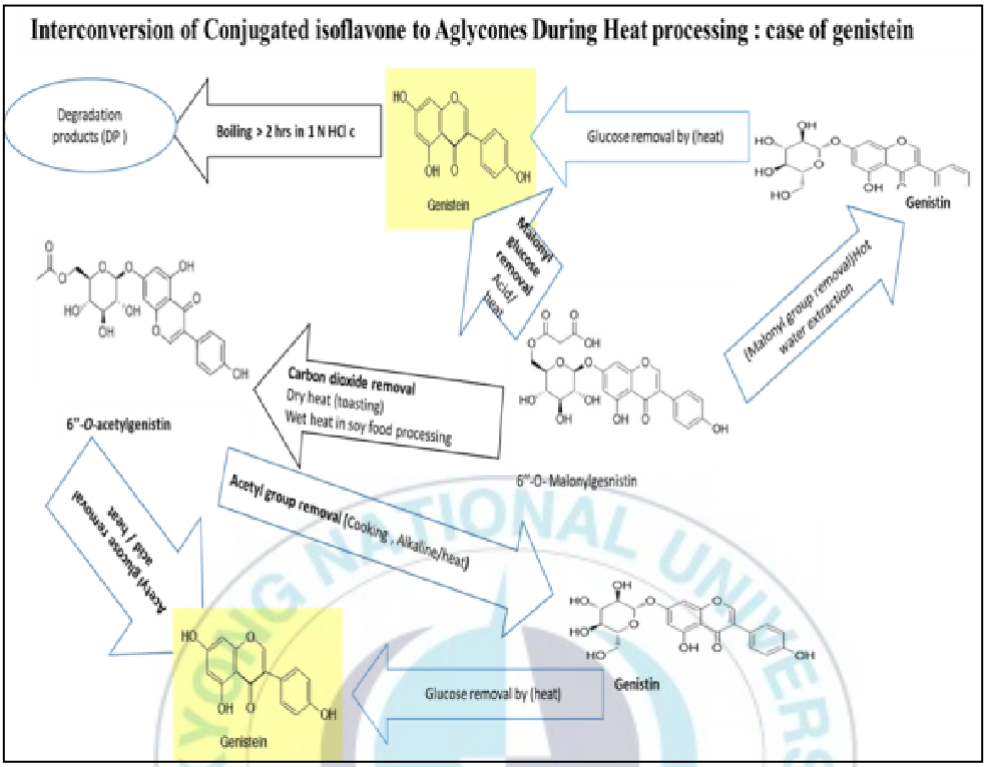


Fig. 1.1. Isoflavones conversions as affected by heat processing, alkaline and acid treatment

The need to extract bioactive compounds from Okara should take into consideration the usefulness of resulting products. The attempts to extract bioactive compounds for commercial benefit and value addition of underutilized byproducts using conventional extraction techniques resulted in products of less quality and unsafe for human consumption. These are caused by the use of organic solvents that are toxic for human being and hence need extra processing steps to remove these solvents which affect the quality of products. The use of novel processing technologies is the key to valorizing these byproducts.

1.3. Subcritical Water Extraction

Subcritical water extraction (SWE) exploits the liquid state at elevated temperatures (100–374 °C) maintained by elevated pressures (1–220 bar) **(Figure 1.3.)**, resulting in water with a dielectric constant equivalent to that of methanol, enhanced diffusivity and mass transfer compared with normal conditions, and solvation properties promoting conditions where both polar and non-polar solutes might be extracted with minimal degradation problems [15]. Singh and Saldaña demonstrated how SWE of potato peel resulted in increased antioxidant activity [16]. Additional examples were provided in the separate reviews of Carr *et al*. and Ko *et al*. [17, 18].

In order to extract isoflavones from okara using subcritical water it is important to optimize the extraction conditions and to characterize the isoflavones profiles. The chemical transformation of isoflavones during subcritical water hydrolysis can never be understood without carrying out the kinetic modelling of their interconversions and

degradations, therefore the second chapter focuses on the optimization and kinetics modelling of isoflavones during subcritical water hydrolysis.

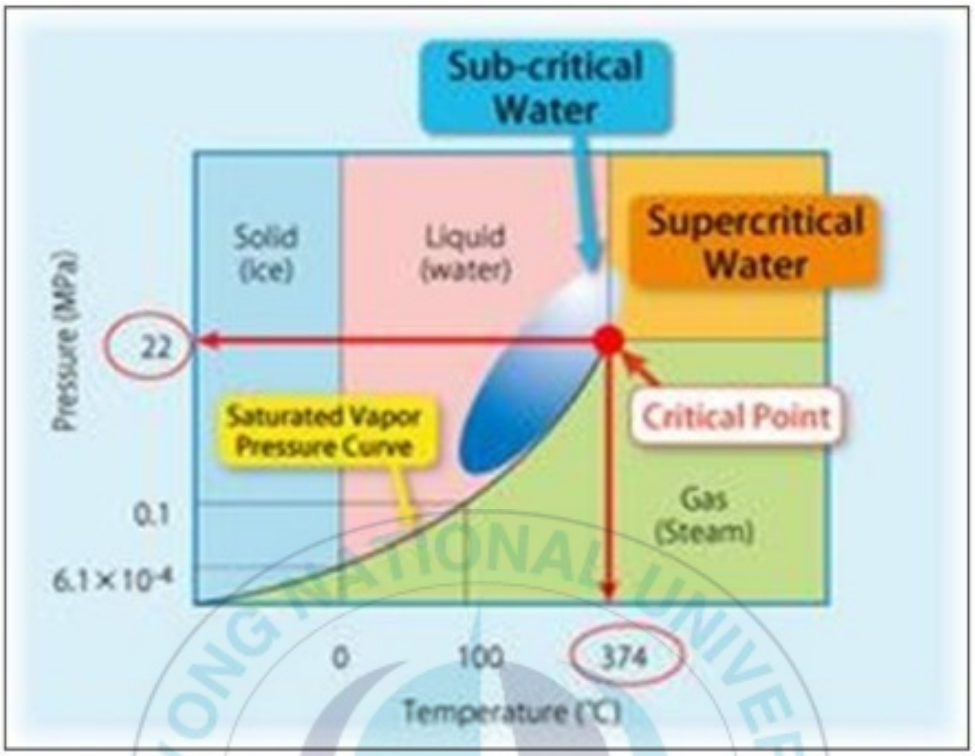


Fig. 1.2. Phase Diagram of Water

CHAPTER 2

Optimization and kinetics modelling of okara isoflavones extraction using subcritical water

2.1. Introduction

Soybean (*glycine max*) is the most extensively grown and consumed legume in the world, rich in proteins and edible oil, and a variety of micronutrients and phytochemicals [1]. Some of the more well-known phytochemicals are isoflavones, which are attractive for further research because their steric structure is equivalent to that of steroidal estrogens, enabling them to bind to human estrogen receptors and employ various estrogenic and anti-estrogenic effects [19]. Isoflavones have also been linked to health benefits, including: retarding the development of prostate cancer and inhibiting the spread of prostate tumors [20]; reduction of menopausal symptoms; prevention of bone fracture by increasing bone mineral density; and, possible cardiovascular health improvements [21].

Isoflavones from soybean are classified into four groups depending on their chemical conformation and the presence of conjugated groups. Three isoflavones, aglycones (genistein (GE), daidzein (DE), and glycitein) are non-conjugated native forms whereas the β-glucosides (genistin (GI), daidzin (DI) and glycitin) are conjugated forms containing a glucose unit. Malonylglucosides, consisting of 6"-o-malonylgenistin (MG), 6"-o-malonyldaidzin (MD), and 6"-o-malonylglycitin and the acetylglucosides 6"-o-acetylgenistin (AG), 6"-o-acetyldaidzin (AD), and 6"-o-acetylglycitin are results of heat processing, converting the malonyl group through

esterification [5]. Compounds MG, GI, and DI account for 83%–95% of total isoflavones in soybean [6]. Despite this abundance, conjugated isoflavones tend to exhibit a weak estrogenic activity [22] and bioavailability when compared with aglycones [23]. The production of potential isoflavones-based nutraceutical and pharmaceutical products, by increasing the release of isoflavone aglycones in the final extracts, has been reported by Yatsu *et al*. who proposed the hydrolysis of soybean isoflavone glucosides to recover and increase the concentration of aglycones using ethanol, heating, and acidic hydrolysis [24].

Subcritical water extraction (SWE) exploits the liquid state at elevated temperatures (100–374 °C) maintained by elevated pressures (1–220 bar), resulting in water with a dielectric constant equivalent to that of methanol, enhanced diffusivity and mass transfer compared with normal conditions, and solvation properties promoting conditions where both polar and non-polar solutes might be extracted with minimal degradation problems [15]. Singh and Saldaña demonstrated how SWE of potato peel resulted in increased antioxidant activity [16]. Additional examples were provided in the separate reviews of Carr *et al*. and Ko *et al*. [17, 18]. Several processing methods have been employed to study the chemical conversions of isoflavones and the results obtained have led to the development of different kinetics models that describe the conversions and degradations of soybean isoflavones in different food processing systems. Chien *et al*. developed a model for the degradation and conversion of isoflavones during both moist and dry heating conditions (100–200°C) [12]. Niamnuy *et al*. investigated the drying kinetics of soybean during gas-fired, infrared heating via hot air using different temperatures [13]. Also, using a circulating-hot-air, oven drying of okara, Muliterno *et al*. revealed the conversion of isoflavone glucosides to aglycones

and a reduction of isoflavones content at 70°C, with the drying kinetics best fitted with Fick and Page diffusion models [14].

Although Zaheer and Humayoun Akhtar showed an increased biological activity of okara after fermentation [25], no studies exist in the current published literature describing SWE of okara leading to enhanced isoflavone aglycones. The overall aim of this work was to define controllable process parameters that would increase significantly the isoflavone aglycones and total phenolics contents of okara extracts, for future use as potential ingredients in nutraceutical industries. The principal aim of the work herein was to further valorize okara byproducts by using SWE to maximize the conversion of the primary isoflavone glucosides and their respective aglycones. To this end, we report the response surface modelling of the variables in the extraction process thereby optimizing the conditions for modeling of the kinetics of conversion of selected isoflavone components.

2.2. Materials and Methods

2.2.1. Chemicals and Reagents

Isoflavone standards, genistin, daidzin, genistein and daidzein (HPLC ≥ 99% purity) were obtained from Extrasynthese, Z.I. Lyon Nord, France, and HPLC grade acetonitrile, water, and methanol from Honeywell, Ulsan, Korea. Acetic acid (99.5% glacial) was supplied by Samchun, Pyeongtaek, Korea, thimble and syringe filters were from Advantec, Japan. All other reagents and standards used in this work were obtained from Sigma-Aldrich (St. Louis, USA) of analytical grade, and used without further purification.

2.2.2. Sample preparation

Dry okara samples with a moisture content ≅ 7% were obtained from Shinangchon, Busan, Korea. Okara byproducts were dried using hot-air oven drying at 50°C and sieved with a 450 μm mesh size to obtain a maximum diameter, dry powder. The moisture content of the powder was determined prior to subcritical water hydrolysis using the method of Jindal and Siebenmorgen [26].

2.2.3. Subcritical water hydrolysis

Okara hydrolysis was made using a laboratory scale Phosentech, MSR-1L-15M-270 subcritical water extractor (Daejeon, Korea), operated in static extraction mode ($100 < \text{temperature} \leq 200°C$, $20 < \text{pressure} \leq 50$ bar, $10 < \text{solid/liquid volume} \leq 30$ mg/mL, 5 min). These values represented the optimization extraction condition ranges, as explained below. Once these conditions were controlled reliably, a varying reaction time was applied (30 min intervals up to 275 min).

2.2.4. Soxhlet extraction of isoflavones from okara

Soxhlet extraction of okara isoflavones was carried out according to the method of Luthria *et al*. [27]. Briefly, an okara sample (3 g) was loaded into the extraction compartment of a soxhlet apparatus and extracted (80% methanol (99% purity), 150 mL, 7 h). After extraction, aliquots of the extracts (1 mL) were filtered (0.45 μm) and analyzed using HPLC. Triplicate extracts and duplicate HPLC analyses were made and the content considered to be the *total* isoflavones content of the extracted okara samples. These totals were the basis values for subsequent SWE analyses.

2.2.5. Optimization and experimental design

Extraction optimization was made using Response Surface Modelling (RSM) with temperature (A, 120–160°C), pressure (B, 20–50 bar), and solid mass: fluid volume ratio (C, 10–30 mg/mL) set as the primary, independent variables, based on preliminary experiments. These variables were set as coded values using the Box-Behnken design method in the range -1 to +1 to normalize each reaction parameter to affect the response evenly. Values for these variables were summarized in Table 1. A one-factor analysis was also conducted. The total isoflavones concentration was the sum of the 4 isoflavones (GI, DI, GE and DE) and the total was considered as the sole factor for the three levels. A second-order regression equation for the Box-Behnken design yielded 15 experimental variations. This design was used to predict the levels of the factors: temperature, A = 146.23°C; pressure, B = 39.8 bar; and mixing ratio, C = 20 mg/mL (Table1). These values were also used to evaluate the extraction variables for the maximum yield of total isoflavones. The response variables were fitted to a second-order polynomial, Eq. (1):

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i<j=1}^{3} \beta_{ij} X_i X_j \quad (1)$$

where Y was the response variable, X_i were independent variables, and β_0, β_i, and β_{ii}, were regression coefficients for the intercept in the linear, quadratic, and interaction mixing-terms. Analysis of Variance (ANOVA) was performed to evaluate any significant differences between independent variables ($p < 0.05$). To illustrate the relationship between the independent variables and their responses, the polynomial regression equation was further expressed using Design Expert statistical software (Version 7.1.3, Stat-Ease, and Minneapolis, MN, USA).

2.2.6. HPLC Isoflavones analyses

Isoflavone analyses were made using an HPLC unit (Hitachi Model-2000 Series, Japan) equipped with a UV-VIS detector (L-2420) and high pressure pump (L-2130). The isoflavone content of each soybean byproduct hydrolysate was determined using the reverse phase HPLC method of Wang and Murphy [10]. Separation set-up conditions were: Mobile phases: Two solutions as (a) acetic acid (0.1% glacial) and acetonitrile in water (2 %), and (b) acetic acid (0.1% glacial) in acetonitrile (100%). Gradient development: Injection of 50 µl with the system automatic sampling of 20 µL. Mobile phase flow was solvent (b), 10% for first 10 min, increased to 14% up to 12 min, 20% up to 20 min, and finally increased to 70% until 32 min flow time, with constant flow rate throughout (1 mL/min). Detector setting: 254 nm for the 32 min running time. Column: Agilent Eclipse plus C_{18} Column (4.6 × 250 mm ID, 5.0 µm particle size). Prior to injection, each hydrolysate aliquot was filtered (0.45 µm). Quantification was achieved using standard addition of each sample isoflavone with concentration defined via linear calibration plots for the standards ($R^2 = 0.9995$), and the resulting sample concentrations expressed as mg/100 g fresh weight. The UV spectra were obtained and developed using EZChrom Elite for Hitachi Version 318 software.

2.2.7. Statistical analyses and kinetics modelling

The multiple and repeat sets of data were analyzed as an ANOVA from IBM SPSS version 20 (SPSS Inc., Chicago, USA). The differences within groups were defined using the Tukey HSD $p < 0.05$. The kinetics modelling of the isoflavones transformations were made by integrating expressions for the species balances using

Laplace transform analyses, with the resulting expressions reproduced in the Supporting Information.

2.3. Results and Discussion

Okara represents a widely available but infrequently considered source of isoflavones. Although efforts to enhance the availability of these biologically active compounds by moist heat processing result in high levels of degradation of the isoflavone glucosides, thereby preventing their conversions to aglycones [12], the resulting relatively high levels of degradation products suggests alternative methods of recovery would potentially rectify this problem. Analysis of the recent investigations into, and optimization of SWE for the extraction of phytochemicals such as total phenolics and total antioxidants [28] suggested that this extraction method would benefit isoflavones recovery from okara.

2.3.1. Variables and response surface optimization

Table 2 summarized the SWE optimization results for isoflavones from okara. The ANOVA results (Table 2.2) indicated strong significance between model and experimental data, with $p < 0.0005$, an insignificant lack of fit $p < 0.4058$, and an F-value 1.61 relative to the pure error, each of which indicated an adequate modelling to predict response from variables' adjustment. A strong correlation existed between the response and changes in independent variables as $R^2 = 0.9857$ were the result of the nominal values for T ($120 < T \leq 160$ °C), P ($20 < P \leq 50$ bar), and α ($10 < \alpha \leq 30$ mg/mL). The optimum conditions for total isoflavones extraction resulted in a second-order model as:

$$\text{Total Isoflavones} = 94.88 + 6.08T - 0.61P - 10.57\,\alpha + 0.69TP - 8.57T\,\alpha - 5.96P\,\alpha - 14.26T^2 - 2.27P^2 - 25.65\alpha^2 \qquad (2)$$

Table 2.1. Box-Behnken RSM design for isoflavones SWE from okara

				Total isoflavones (mg/100g)		
Run	T, ºC	P, bar	α, mg/ml	Actual	Predicted	%, R
1	120	25	20	74.77	66.35	63.50
2	160	25	20	68.44	95.38	58.12
3	120	50	20	73.16	74.40	62.13
4	160	50	20	69.71	53.31	59.20
5	120	37.5	15	67.09	77.73	56.98
6	160	37.5	15	78.05	77.05	66.28
7	120	37.5	25	66.04	95.38	56.08
8	160	37.5	25	55.57	70.08	47.19
9	140	25	15	94.64	69.35	80.37
10	140	50	15	95.78	70.97	81.34
11	140	25	25	83.96	70.64	71.30
12	140	50	25	75.17	84.01	63.84
13	140	37.5	20	93.35	95.72	79.28
14	140	37.5	20	94.77	95.38	80.48
15	140	37.5	20	98.00	92.75	83.23

%R=T.I*100(SWE)/T.I (soxhlet, 80% Methanol)

Table 2.2. Analysis of variance (ANOVA) of the fitted second-order polynomial model for total isoflavones

Source	Sum of Squares	df	Mean Square	F-Value	p-value	
Model	2451.04	9	272.34	35.21	0.0005	significant
Residual	38.68	5	7.74			
Lack of Fit	27.34	3	9.11	1.61	0.4058	not significant
Pure Error	11.34	2	5.67			
Total	2489.72	14				

The overall response of the solubility of the total isoflavones with the varying conditions could also be linked with solvent density, a primary variable in extraction modeling. In the RSM analyses the solvent phase density would range from $910.3 < \rho \leq 944.5$ kg/m^3, responding to the T and P conditions: (160°C, 50 bar) and (120°C, 20 bar), respectively. Density changes from normal conditions to extraction conditions were accounted for when the liquid volumes in the solid-extraction fluid volume ratio were defined. An interesting, but intuitively understandable improvement also comes with a decrease in mixing ratio [29]; more solvent with less solute would appear to give increased solubility, or extraction capability. Factor analysis at the optimum mixing ratio endorses this observation, as well as it having been made elsewhere; Rostagno *et al*. found that the extraction efficiency of isoflavones increased constantly when the sample size was reduced [30].

Richter *et al*. suggested an increase in solubility would generally equate with an improved mass transfer (as desorption) from the solid phase into the solvent (bulk) phase [31]. Such conditions would be promoted by enhanced solvent-solute interactions, defined by the combination of van der Waals (as dispersion) forces and hydrogen bonding leading to, or even due to, dipole-dipole and/or (solvent-dipole)-(solute induced dipole) interactions. Essentially, the chemical potential caused by this combination of interactions would exceed the chemical potential caused by similar forces within the adsorbed phase, leading to a net negative Gibbs energy for solvation. If pure isoflavones were involved in these thermodynamics descriptions, the energy could be deduced from enthalpy measurements [32]. In the current work, the isoflavones as GI and GE, and DI and DE, were simultaneously extracted and mixtures tend to give non-linear solubility curves with temperature (and solvent density)

analyses, hence, the optimization curves exhibited broad sloping gradients for each pair of variables with the third constant. The optimum conditions for the mixed isoflavone extractions were T = 146.23°C, P = 39.8 bar, and α = 20mg/ mL, represented by solvent density 921.9 kg/m^3 (Fig.2.1a-c).

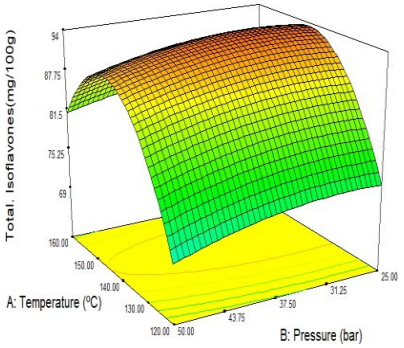


Fig. 2.1a: Response surface plot for isoflavone recovery as $f(T, P)$ constant α

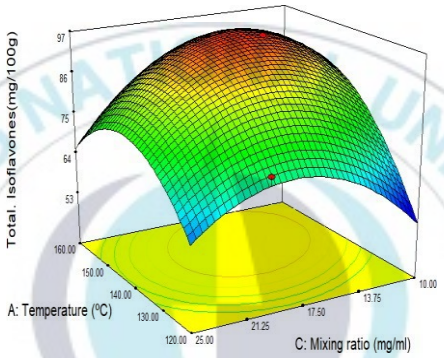


Fig. 2.1b: Response surface plot for isoflavone recovery as $f(T, \alpha)$ constant P

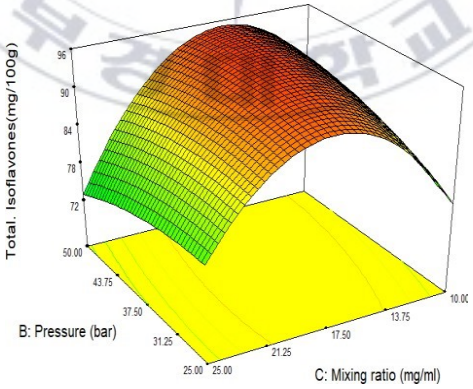


Fig. 2.1c: Response surface plot for isoflavone recovery as $f(\alpha, P)$ constant T

2.3.2. Effect of single and interaction of factors on the response

The effects of *T, P*, and α on the recovery of total isoflavones (regarded as response) were shown in the Fig 2.1a-c. Each factor employed during the optimization of isoflavones recovery via SWE was investigated for its effect on extraction and, as reported in recent reviews, temperature was identified as (one of) the key parameters affecting efficiency. This conclusion was based on the change in value of the water phase dielectric constant with the temperature and pressure from 78.36 (25ºC, 1 bar) to 18.53 (350ºC, 150 bar) [33]. Clearly the increased temperature conditions used in SWE converts water to a low polarity fluid mimicking an organic solvent, with minor extractant degradation problems [34].

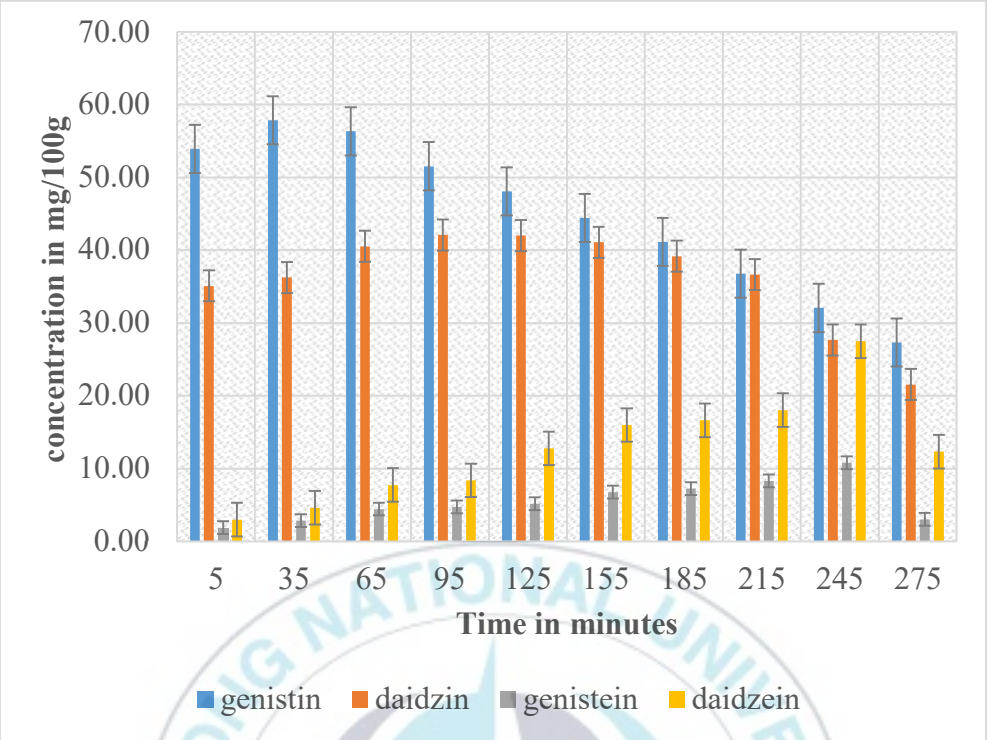


Fig. 2.2. Isoflavones aglycones and β-glucosides as over the reaction times

2.3.3. Effect of Temperature, Pressure and mixing ratio

The SRM analyses provided useful background information and overall direction for process optimization, and provided the basis for isoflavones extraction during hydrolytic de-esterification. Total isoflavones recovery, summarized as the "response" to variation in the process variables were shown in Fig.2.1a-c. Temperature changes had a compelling impact on recovery, for reasons commented on above (Table 2.1). An increase in extraction temperature from 120 → 140°C raised the overall recovery from 60% to 77.12 %.(soxhlet extraction via the 80% methanol-water as basis for conventional method for isoflavones recovery). This observation was consistent with those of Li-Hsun *et al*. who used the steepest ascent design for their optimized SWE of total isoflavones from defatted soybean flakes, and showed an improved efficiency at 110ºC and 45 bar where the calculated dielectric constant of water would be 53.30 from 78.at ambient temperature [35]. However it should be noted that, a further increase in temperature to 160ºC, where the dielectric constant would be 42.50, reduced the yield of the total isoflavones from 77.12 to 57.70% (average), suggesting that the dielectric constant of water was not a major factor supporting extraction. The decreased recovery would primarily be attributed to an increased thermal degradation kinetics [29].

Temperature change also has a greater influence than pressure change on solvent density. As noted above, the solvent density at optimum SWE conditions would be calculated as 922.47 kg/m^3, decreasing by 1.4% to 909.46 kg/m^3 (160°C, 40 bar). The extractant density in Li-Hsun's work would be 953.04 kg/m^3 (110°C, 45 bar) was similar to that at the lowest temperature in the SRM analyses at 944.99 kg.m^3 (120°C, 39.8 bar) decreasing to 928 kg.m^3 at 140°C, 39.8 bar and 909.45 kg.m^3 at 160°C, 39.8 bar.

In conclusion, the elevated thermophysical conditions promoted extraction due to a combination of reduced polarity and density of the solvent phase. From the results in Fig. 2.1a-c, one could conclude that changes in pressure had negligible effects on the total amount of isoflavones recovered, implying that both the reaction process and the reactants and products were nominally unresponsive to fluid pressure. It would also be accurate to conclude that water was only a marginal contributor to the reaction process, again interpreted in terms of relatively small fluid density changes across the pressure range examined, as also observed recently [36]. The SRM-optimized extraction pressure was 39.8 bar. The contribution of the mixing ratio variable to process optimization, as solids mass per milliliter extractant, addressed overall isoflavones solubility. The recovery response to changes in mixing ratio were shown in combination with temperature (Fig. 2.1b) and with pressure (Fig.2.1c). Recovery increased with increase in solids mass from 10 mg/mL to 20 mg/mL however, above this proportion, the total isoflavones recovered as a fraction of the initial solids decreased. Rostagno *et al*. made similar observations from SWE for isoflavones noting that extraction efficiency increased on decreasing sample mass from 0.5 to 0.05 g [30]. The solubility optimum condition was also defined via SRM to be 20 mg/mL.

2.3.4. Extraction Efficiency and isoflavones profiles

The preliminary extraction investigations in preparation for SRM included a time element (5 min) (Table 2.1). The optimized conditions (140°C, 37.5 bar, 20 mg/mL) yielded 83% of available isoflavones compared with the basis assumption of 100% soxhlet extraction. This SWE amount compared favorably with SWE of defatted soybeans (100ºC 48 bar, 3 h) [35], with the difference between the SWE attributed to the difference in extraction times, 5 min compared with 180 min.

Scheme 1 and Fig. 2.3 showed proposed hydrolytic conversion pathways for the β-glucosides (GI and DI) to their respective aglycones (GE and DE). As a result of both extraction and reaction, these four compounds were classified as the total isoflavones. The amounts of these extracted under the SRM-optimized conditions were further examined using time as the variable (Table 2.2).

Table 2.3. Concentration of isoflavones via SWE and soxhlet extraction

	β-Glucoside		Aglycones			
Time (min)	GI	DI	GE	DE	T.I	% R
5	53.97±0.04 [a]	35.14±0.045 [e]	1.70±0.43[i]	3.04±0.02[j]	93.85±0.53[g]	80.21
35	57.87±0.02 [b]	36.31±0.03 [d]	2.90±0.04[g]	4.70±0.05[i]	101.78±0.14[e]	86.44
65	56.38±0.03 [c]	40.56±0.01 [b]	4.48±0.03[f]	7.85±0.07[h]	109.27±0.14[a]	92.8
95	51.77±0.30 [d]	42.13±0.06 [a]	4.81±0.04[f]	8.48±0.09[g]	107.19±0.49[c]	91.03
125	48.21±0.11 [e]	42.08±0.04 [a]	5.23±0.06[e]	12.91±0.08[e]	108.43±0.29[b]	92.08
155	44.42±0.03 [f]	41.09±0.03 [b]	6.8±0.04[d]	16.05±0.03[d]	108.36±0.12[b]	92.03
185	41.18±0.02 [g]	39.22±0.03 [c]	7.30±0.02[e]	16.70±0.04[e]	104.4±0.11[d]	89.23
215	36.85±0.09 [h]	36.71±0.06 [d]	8.37±0.04[h]	18.10±0.05[b]	102.02±0.24[e]	86.64
245	32.18±0.11 [i]	27.90±0.27[f]	10.96±0.1[a]	27.53±0.05[a]	98.57±0.53[f]	83.71
275	27.33±0.13 [j]	21.69±0.78[g]	3.14±0.1[g]	12.45±0.09[f]	64.61±1.1[h]	54.87
Sox, 80% MetOH	65.22±0.5	44.72±0.3	7.81±0.2	0.00	117.75±1	

GI = genistin, DI = daidzin, GE = genistein and DE = daidzein; T.I = Total isoflavones (mg/100g), % recovery over the reaction time Sox, 80%MetOH = Soxhlet Methanol
% R = Percentage recovery = T.I * 100 * (SWE)/T.I (Soxhlet, 80% Methanol)

The predominant compounds extracted in the initial 5 min were the β-glucosides, 95% of total recovered, with the aglycones increasing in their contribution to the total with increasing extraction time. A maximum in amount recovered was achieved at 125 min (92.08%) (Considering soxhlet extraction with the 80% methanol-20% water as basis; 100%). This SWE amount compared favorably with the 93.64% above, at 180 min SWE. The extract composition was 83.27% β-glucosides and 16.73% aglycones, compared with 95% and 5% after 5 min. Rostagno *et al*. demonstrated that malonylgenistin was thermally sensitive at 100°C and further exacerbated at 150°C, leading to GI formation via thermal degradation [30]. Their analyses suggested that β-glucoside to aglycones conversion required 200°C conditions, inconsistent with those reported herein; their results suggested that aglycones formation occurred up to 95 min reaction via the malonyl and acetyl β-glucosides, consistent with previous observations [37], and summarized in Fig. 2a and 2b. Reaction and extraction times beyond 95 min resulted in cessation of β-glucoside formation indicating that the malonyl form degradation had also ceased, while the aglycones concentration continued to increase at the expense of the β-glucosides. The relatively short reaction times monitored at 150°C may have been a reason for why these former reactions were not observed by Rostagno *et al*.; they were observed at 200°C. An additional interpretation of their results would be that the elevated temperature (200°C) contributed to lower selectivity in isoflavones extraction [38]. Similar observation were reported by Moras *et al*. for isoflavones recovery from soybean flour and soybean protein where, at temperatures above 160°C malonyl and other conjugated isoflavones were rapidly degraded but aglycones concentrations increased. These changes also affected the overall isoflavones recovery efficiency, defined as amount of isoflavones per 100g solid phase, suggesting 150-160°C would be a critical extraction temperature selection during

process optimization [39]. This efficiency observation was consistent with those of Rostagno's recoveries at 150°C compared with 200°C. These conclusions support the optimum extraction temperature defined by the current SRM, 146.23°C.

In summary, each of the above analyses of the current and previous researches and their conclusions support the optimized (T, P, α) conditions as the basis for SWE reaction kinetics analyses. Additionally, the various times, temperatures, and compounds recovered warranted a more detailed reaction kinetics analysis.

2.3.5. Effect of extraction time on isoflavones

Chien *et al*. produced a detailed schematic correlation from the moist and dry heating conditions and resulting isoflavones recovered [12], similar to those recovered via the current work summarized by Fig. 2.1. Clearly, for both species GE and DE formation the primary precursor GI and DI would be formed by simultaneous hydrolytic de-esterification of MG and AG for GI, or MD and AD for DI.

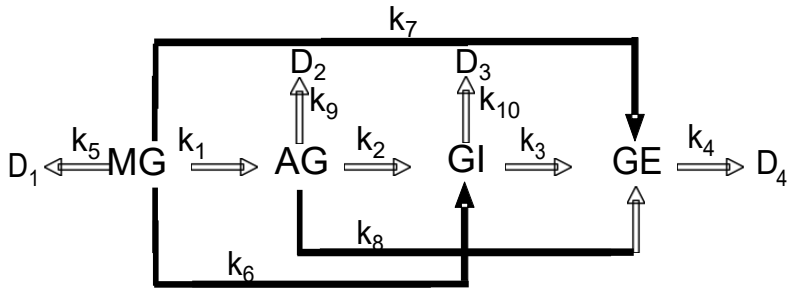


Fig. 2.3a: Schematic of conversion and degradation paths during SWE

MG = Malonylgenistin; AG = Acetylgenistin; GI = Genistin; GE = Genistein

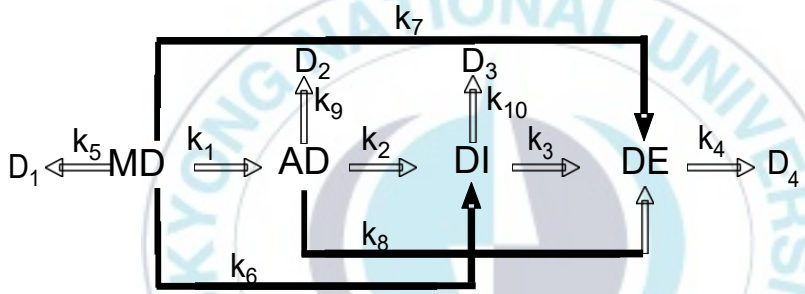


Fig. 2.3b: Schematic of conversion and degradation paths during SWE

MG = Malonyldaidzin; AG = Acetyldaidzin; GI = Daidzin; GE = Daidzein

Note: D_x = undefined degradation product; k_n = rate constant for each reaction, n. Also, D_x and k_n in Fig. 2a would not be same product or value in Fig. 2b

The overall time dependent isoflavones concentrations were summarized in Fig. 3, focusing on GI and GE in Fig. 2.4a and DI and DE in Fig. 3b, with units of mmole/100 g solid (okara) allowing interspecies comparison. The general shape of the GE and GI curves were reflected in Fig. 2.4b for DI and DE. The significant difference between the sets of data was the time position of the maximum for GI and DI. In contrast, with the maximum accumulation of GI occurring up to 50 min extraction, that for DI required 150 min, suggesting that the rates of hydrolytic de-esterification of MG and AG would be greater than those for MD and AD, or the rate of formation of (adverse) degradation products from MD and AD exceed those from MG and AG. This observation remained obscure since degradation products were not quantified. Further comparison between the two data sets indicated that the amount of DE accumulated exceeded that for GE, an observation consistent with Luthria *et al*. who showed concentrations of DE exceeded all other isoflavones recovered from soybeans during pressurized liquid extraction [27].

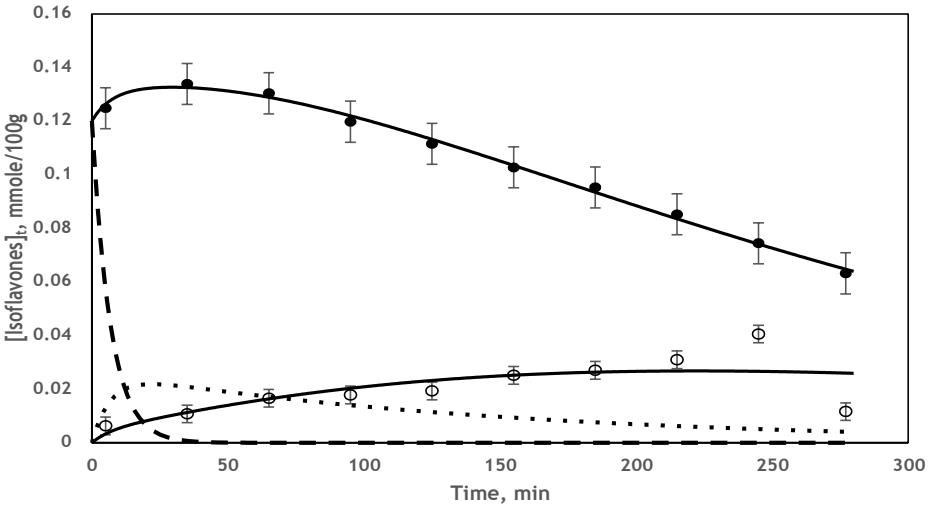


Fig. 2.4a. Time dependent Genistin (●) and Genistein (○) accumulation

Ɪ = standard deviation of 3 measurements; — = modelled $[GI]_t$ and $[GE]_t$; - - - modelled $[MG]_t$ decay; • • • modelled $[AG]_t$

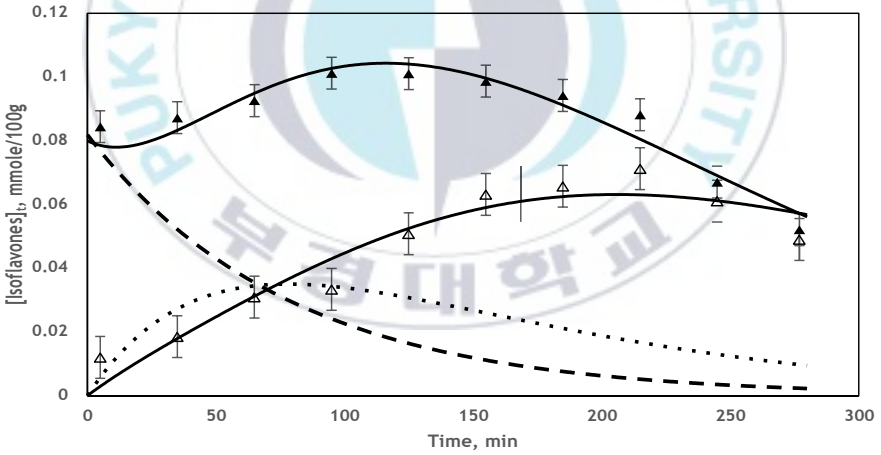


Fig. 2.4b. Time dependent Daidzin (▲) and Daidzein (Δ) accumulation

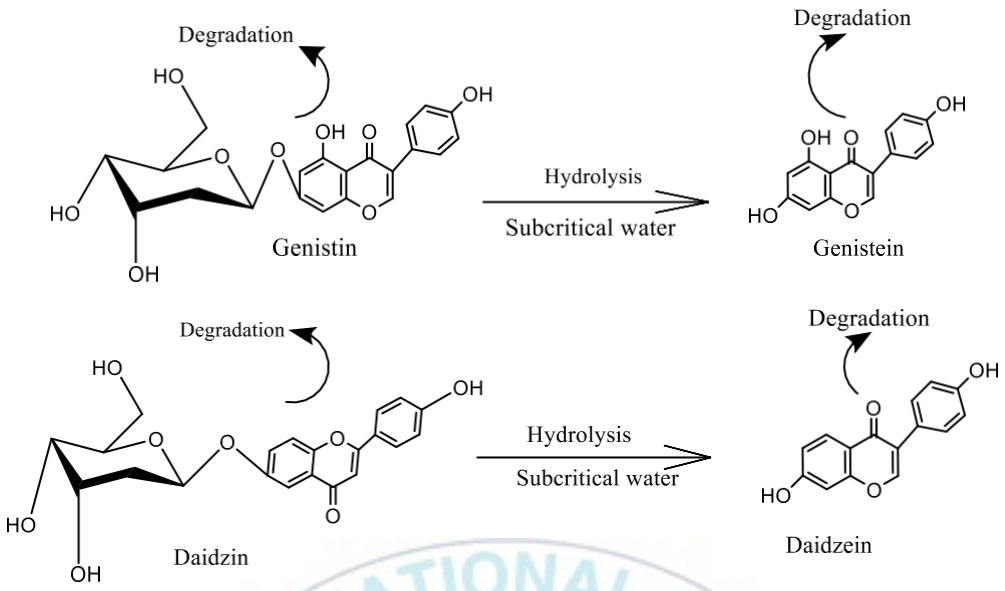
Ɪ = standard deviation of 3 measurements; — = modelled $[DI]_t$ and $[DE]_t$;

- - - modelled $[MD]_t$ decay; • • • modelled $[AD]_t$

The previous analyses of Chien *et al*. focused on dry and moist heat extraction conditions (T = 100, 150 or 200°C, P = 1.01 bar) to simulate various food preparation or processing conditions. Their results demonstrated a modest resistance to hydrolytic de-esterification in dry heat conditions for MG → AG; the conversions of GI → GE (and MG → GE) were identified as the rate determining steps across the hydrolysis process. The presence of a water vapor atmosphere ameliorated the reaction rates for each pathway, but also assisted degradation product formation. The apparently high initial GI concentration within 5 min of the current SWE processing was consistent with the observations of Chien *et al*. who showed rapid decomposition of MG and AG to GI with increasing temperature, from 100 → 150 → 200 °C, in a moist atmosphere. Although each β-glucoside would be responsible for GE formation, the principal mechanism would be via GI hydrolysis, thus, the primary focus of the current work was on GI and GE. Similar arguments and observations exist for the conversion and recovery of DI to DE.

2.3.6. Isoflavones reaction kinetics modelling

Considering the optimized SWE conditions (T = 146.23°C, P = 39.8 bar, α = 20 mg/ml) reaction pathways for GI to GE in Fig. 2.3a and, as a parallel discussion for DI to DE (Fig. 2.3b), changes in the isoflavone β-glucosides (GI or DI) and aglycones (GE or DE) compositions would, most probably, follow the reactions summarized in Scheme 1:



Scheme 1: Probable reaction mechanisms for β-glucoside decomposition and aglycones formation

Although only accumulated amounts for GI and GE (and DI and DE) were considered, the kinetics modelling for GE formation must also include its other two precursors, MG and AG, otherwise the relationship between GI and GE reduces to an overly simplified pseudo-first order kinetics, with poor data reproduction from the subsequent model. Individual component material balance analyses were necessary to provide detailed rate expressions, with those for GI and GE resembling those developed by Chien *et al*. Subtle differences were formulated since the degradation reaction processes were considered in the current work but not included in the previous research. Similar rate expressions were developed and applied to the DI and DE systems based on Fig. 2.3b. Details of the rate equations and their solutions as reaction time-dependent species concentrations were prepared for GI and GE, and for DI and DE and the summary of the expressions are given in Eq. (3) – (10).

Consider MG balance and kinetics:

$$\frac{d[MG]}{dt} = -k_1[MG] - k_5[MG] - k_6[MG] - k_7[MG] = -(k_1 + k_5 + k_6 + k_7)[MG] \quad (3)$$

This would be equivalent to simple first order kinetics:

$$[MG]_t = [MG]_0 \exp(-K_{1567}t) \quad (4)$$

Consider AG balance and kinetics:

$$\frac{d[AG]}{dt} = k_1[MG] - k_2[AG] - k_8[AG] - k_9[AG]$$
$$= k_1[MG] - (k_2 + k_8 + k_9)[AG] \quad (5)$$

Substitution of Eq. (4) into Eq. (5) collecting terms and integrating the resulting Pfaffian integral gives:

$$[AG]_t = \frac{k_1}{K_{289} - K_{1567}}[MG]_0 \left(\exp(-K_{1567}t) - \exp(-K_{289}t)\right) \quad (6)$$

Consider GI balance and kinetics:

$$\frac{d[GI]}{dt} = k_6[MG] + k_2[AG] - k_3[GI] - k_{10}[GI]$$
$$= k_6[MG] + k_2[AG] - (k_3 + k_{10})[GI] \quad (7)$$

Substituting Eq. (4) and Eq. (6) into Eq. (7) results in:

$$\frac{d[GI]}{dt} = \frac{k_1 k_2}{K_{289} - K_{1567}}[MG]_0 \left(\exp(-K_{1567}t) - \exp(-K_{289}t)\right)$$
$$+ k_6[MG]_0 \exp(-K_{1567}t) - K_{310}[GI] \quad (8)$$

Consider GE balance and kinetics:

$$\frac{d[GE]}{dt} = k_7[MG] + k_8[AG] + k_3[GI] - k_4[GE] \tag{9}$$

Substituting Eq. (4) and (6) into Eq. (9) gives

$$\frac{d[GE]}{dt} = \frac{k_1 k_8}{K_{289} - K_{1567}}[MG]_0\left(\exp(-K_{1567}t) - \exp(-K_{289}t)\right) + k_7[MG]_0\exp(-K_{1567}t) + k_3[GI] - k_4[GE] \tag{10}$$

$$[GI]_t = \frac{\left([MG]_0 \exp(-K_{1567}t)(K_{289}k_6 - K_{1567}k_6 + k_1k_2)\right)}{\left((K_{289} - K_{1567})(K_{310} - K_{1567})\right)} - \frac{\left(\exp(-K_{310}t)K_{310}^2[GI]_0 - K_{310}K_{310}[GI]_0 + K_{289}K_{310}[GI]_0 - K_{289}[MG]_0 k_6 - K_{310}[MG]_0 k_6 + [MG]_0 k_1k_2\right)}{\left((K_{289} - K_{310})(K_{310} - K_{1567})\right)} + \frac{\left([MG]_0 k_1k_2 \exp(-K_{289}t)\right)}{\left((K_{289} - K_{310})(K_{289} - K_{1567})\right)} \tag{11}$$

$$
\begin{aligned}
[GE]_t = {} & \frac{\left(\begin{array}{l} \left(K k^2[GE]_0 - k^3[GE]_0 + K k^2[GE]_0 + K k^2[GE]_0\right) \\ \left(+k k^2[GI]_0 + [MG]_0 k^2 k + K K K [GE]_0 - K K k [GE]_0\right) \\ \left(-K_{289}K_{1567}k_4[GE]_0 - K_{310}K_{1567}k_4[GE]_0 + K_{289}K_{1567}k_3[GI]_0\right) \\ \left(\exp(-k_4 t) + K K [MG]_0 k - K k k [GI]_0 - K k k [GI]_0\right) \\ \left(+K [MG]_0 k k - K [MG]_0 k k + K [MG]_0 k k\right) \\ \left(-K_{310}[MG]_0 k_4 k_7 + [MG]_0 k_1 k_2 k_3 - [MG]_0 k_1 k_4 k_8 - [MG]_0 k_3 k_4 k_6\right) \end{array}\right)}{\left((K_{289}-k_4)(K_{310}-k_4)(K_{1567}-k_4)\right)} \\
& - \frac{\left(\begin{array}{l} \left([MG]_0 \exp(-K_{1567}t)\right) \left(K^2 k + K K k - K K k + K k\right) \\ \left(+K_{289}k_3k_6 + K_{310}k_1k_8 - K_{1567}k_1k_8 - K_{1567}k_3k_6 + k_1k_2k_3\right) \end{array}\right)}{\left((K_{289}-K_{1567})(K_{310}-K_{1567})(K_{1567}-k_4)\right)} \\
& + \frac{\left(\begin{array}{l} \left(k \exp(-K t)\right) \left(K^2 [GI]_0 - K K [GI]_0 + K K [GI]_0\right) \\ \left(K K [GI]_0 + K [MG]_0 k - K [MG]_0 k + [MG]_0 k k\right) \end{array}\right)}{\left((K_{289}-K_{310})(K_{310}-K_{1567})(K_{310}-k_4)\right)} \\
& - \frac{\left([MG]_0 k_1 \exp(-K_{289}t)(K_{310}k_8 - K_{289}k_8 + k_2k_3)\right)}{\left((K_{289}-K_{310})(K_{289}-K_{1567})(K_{289}-k_4)\right)}
\end{aligned}
\tag{12}
$$

Since both isoflavones followed the same conversion and degradation pathway DI and DE will be:

$$
\begin{aligned}
[DI]_t = {} & \frac{\left([MD]_0 \exp(-K_{1567}t)(K_{289}k_6 - K_{1567}k_6 + k_1k_2)\right)}{\left((K_{289}-K_{1567})(K_{310}-K_{1567})\right)} \\
& - \frac{\left(\begin{array}{l} \left(\exp(-K t) K^2 [DI]_0 - K K [DI]_0 + K K [DI]_0\right) \\ \left(+K_{289}[MD]_0 k_6 - K_{310}[MD]_0 k_6 + [MD]_0 k_1k_2\right) \end{array}\right)}{\left((K_{289}-K_{1567})(K_{310}-K_{1567})\right)}
\end{aligned}
$$

$$\left((K_{289}-K_{310})(K_{310}-K_{1567})\right)$$

$$+\frac{\left([MD]_0 k_1 k_2 \exp(-K_{289}t)\right)}{\left((K_{289}-K_{310})(K_{289}-K_{1567})\right)} \quad (13)$$

$$
\begin{aligned}
[DE]_t = {} & \frac{\left(\begin{array}{l}\left(K k^2[DE]_0 - k^3[DE]_0 + K k^2[DE]_0 + K\ k^2[DE]_0\right) \\ \left(\begin{array}{l}+k k^2[DI]_0 + [MD]_0 k^2 k + K\ K\ K\ [DE]_0 - K K k [DE]_0 \\ -K_{289}K_{1567}k_4[DE]_0 - K_{310}K_{1567}k_4[DE]_0 + K_{289}K_{1567}k_3[DI]_0 \\ +K\ K\ [MD]_0 k - K\ k k [DI]_0 - K\ k k [DI]_0 \\ +K^{289\,310}[MD]_0 k k - K^{289\,3\,4}[MD]_0 k k + K^{1567\,3\,4}[MD]_0 k k \\ -K_{310}[MD]_0 k_4 k_7 + [MD]_0 k_1 k_2 k_3 - [MD]_0 k_1 k_4 k_8 - [MD]_0 k_3 k_4 k_6\end{array}\right)\end{array}\right)}{\left((K_{289}-k_4)(K_{310}-k_4)(K_{1567}-k_4)\right)} \\
& - \frac{\left(\begin{array}{l}\left(K^2\ k + K K k\ \square K\ K - K\ K\ k\right) \\ \left([MD]_0 \exp(-K_{1567}t)\right)\left(+K_{289}k_3k_6 + K_{310}k_1k_8 - K_{1567}k_1k_8 - K_{1567}k_3k_6 + k_1k_2k_3\right)\end{array}\right)}{\left((K_{289}-K_{1567})(K_{310}-K_{1567})(K_{1567}-k_4)\right)} \\
& + \frac{\left(\begin{array}{l}\left(K^2\ [DI]_0 - K K\ [DI]_0 + K\ K\ [DI]_0\right) \\ \left(k \exp(-K t)\right)^{310}\left(\begin{array}{l}\square K\ K\ [DI]_0 + K\ [MD]_0 k - K\ [MD]_0 k + [MD]_0 k k \\ +\left(310\,1567\ 0 - 289\ 0\ 6 - 310\ 0\ 6\ 0\,1\,2\right)\end{array}\right)\end{array}\right)}{\left((K_{289}-K_{310})(K_{1567}-K_{310})(K_{310}-k_4)\right)} \\
& - \frac{\left([MD]_0 k_1 \exp(-K_{289}t)\right)\left(K_{310}k_8 - K_{289}k_8 + k_2k_3\right)}{\left((K_{289}-K_{310})(K_{289}-K_{1567})(K_{289}-k_4)\right)}
\end{aligned}
\tag{14}
$$

The time dependent concentration expressions for GI and GE (and DI and DE) were developed by solving the rate equations for MG, AG, GI, and GE as a set of simultaneous equations via Laplace transformation, using MatLab® code (in symbolic format). The expressions for $[GI]_t$ and $[GE]_t$ (and $[DI]_t$ and $[DE]_t$) were complex, but have been reproduced in the Supp. Info. The coefficients K_{xyz} were abbreviations for summation contributions of the rate constants k_x, k_y, and k_z for reaction steps x, y, and z. Numerical solution of the rate expressions rendered values for each K and k in the

expressions; from the K_{xyz} values, individual values for k_x, k_y, and k_z were defined. The expressions for $[GI]_t$ and $[GE]_t$ contain 11 variables to be defined numerically; such a large number could lead to unstable solutions whereby the final values arrived at by minimisation would be initial value dependent. To address and resolve this issue, analyses were made via Solver using the decreasing slope (GRG Nonlinear) method

using variously selected initial values, and compared with results obtained from MatLab code using the nlinmultifit® subroutine, using similar and different initial values. Although the results were not exact values for each coefficient, the rate constants reported in Table2.3 were an average with their standard deviation. The curves shown in Fig 2.4a for GI and GE, and in Fig. 2.4b for DI and DE, were developed using these averaged rate constants. Overall, excellent fits were obtained for both "reactants" and "products", implying an appropriate accounting for the subordinate MG and AG (and MD and DG) compositions and their associated rate constants, even though these were not monitored. The appropriate expressions and rate constants were also used to model the time dependent concentrations for these β- glucoside. The rate constants relevant to GI and GE and their degradation mechanisms were compared (Table 2. 3) with similar analyses made by Chien *et al*., with the latter's results converted from units of h^{-1} to min^{-1} for clarity. For completeness, similar rate constants for DI and DE were included.

Table 2.4. Summary of the rate constants for the degradation and conversion mechanisms

Rate constant k (min^{-1})		Min SSE	M.H ,150 k (min^{-1})	D.H, 150 k (min^{-1})	M.H ,200 k (min^{-1})	D.H , 200 k (min^{-1})
k_3	0.00025	3.44E-06	-	-	-	0.942
k_{10}	0.005	4.67E-05	73.8	2.208	696	276.6
k_4	0.0091		331.8	2.028	756	51.18
k_3	0.0057	4.68E-05				
k_{10}	0.0063	1.98E-04				
k_4	0.005					

M.H: Moist heating; D.H: dry heating – Chien *et al*.

2.3.7. Stability and conversions of isoflavones during SWE

Since the reaction rate expressions for $[GI]_t$ and $[GE]_t$ (and $[DI]_t$ and $[DE]_t$) and their respective degradation products included rate constants for each pathway, $[MG]_t$ and $[AG]_t$ (or $[MD]_t$ and $[AD]_t$) could be evaluated as a proportional dependence on the initial $[GI]_{t=0}$ and $[DI]_{t=0}$. Their concentration-time profiles were included in Fig. 2.4a and 2.4b.

The rapid decay in $[MG]_t$ and was defined by its decomposition, forming $[AG]_t$ and $[GI]_t$ and an undefined degradation product. The $[MG]_t$ decayed to $< 5 \times 10^{-5}$ mmole/100 g (okara) within 55 min, a time consistent with previously reported details discussed above [30] [12] [38]. The relatively shorter time for the decay in the current work demonstrated how SWE promoted hydrolytic de-esterification. The $[AG]_t$ increased at the expense of $[MG]_t$ but was concurrently decomposed to GE and GI, as well as its own undefined degradation product. The competing reactions reached a maximum at approximately 22 ± 2 min, well before MG consumption. The curves describing the measured values for $[GI]_t$ and $[GE]_t$ were well-fitted by their derived rate expressions which contained 10 rate constants and for $[MG]_{t=0}$ as variables. The rate constant for GE → GI in this work compares favorably with that reported by Chein *et al*. [37]. Both the current and their work considered subordinate formation reaction rate constants, but they omitted details of some degradation products. The largest contributor to differences in rate constants and thus subtle differences in curve shapes were in the experimental approaches. Their work heated samples of nominally small mass fluids whereas a heat balance analysis of the current work would also need to consider okara particle heat capacity, heat transfer, cell rupture, and desorption energies and processes. Since the extraction was a non-equilibrium process, each of these

variables would contribute simultaneously to reactant release and decomposition. Formation of GI reached a maximum as a decomposition product of MG and AG at 29 ± 2 min. Clearly, at times exceeding this value the rate of GE formation predominated. The general behavior of the reactants and products herein was consistent with the reports from Chien *et al*. but differences in time at 150°C would again be attributed to the experimental conditions.

The trends of each reactant and product curves for the daidzin-based *β*-glucosides and aglycones were a reflection of those in Fig. 2a. Differences were clearly in the rate of decay of MD and the larger amounts of AD formed. Here, it was more apparent that the rate determining step of the entire reaction sequence was the decomposition of AD since maxima in $[DI]_t$ and $[DE]_t$. occurred at times exceeding $[AD]_{max} = 78 \pm 2$ min. The curve fittings in Fig. 3a and 3b suggested that the $[GE]_t$ and $[DE]_t$ would increase continuously with reaction time. The decreasing concentrations in amounts extracted suggests aglycones decomposition; maximum $[GE]_t$ occurred at t = 222 ± 2, and for $[DE]_t$, t = 205 ± 2 min. Thus the average time, $t_{max,\ average} = 213.5 \pm 8.7$ min equates to an optimum maximum extraction time.

In the extraction works of Moras *et al*. and Rostagno *et al*., soybean precursor heat capacity appears to be an explanation for differences in their observations compared with the current work. Each of their conclusions were made for short time analyses, ,20 min, and different thermal conditions. In some cases, they were 100°C and others 150, 160 or 200°C [30] [39]. Thermal stability analyses made by Yue *et al*. further endorse our conclusions and also indicate that a reaction enthalpy of activation exists for GE → GI and DE → DI, which may also be combined with desorption enthalpies;

rates of degradation at 150°C were 3-times the rate at 100°C, and considerably enhanced at 200°C. These observations were endorsed recently by Andrade *et al*. [40].

Clearly, considerable efforts have been made to study the thermal conversion of conjugated isoflavones to aglycones, focusing on high temperatures up to 200°C. Such conditions detract from optimum yields. The proposed optimum conditions, defined by maximum yields of isoflavone aglycones with minimized degradation products, would be via SWE at T = 146.23°C, P = 39.8 bar, α = 20 mg/mL, and t_{max} = 213.5 min.

2.4. Conclusion

The SWE of isoflavones from soybean particles was developed after a surface response modeling of the operation variables temperature, pressure, and solid-extractant volume. Soxhlet extraction of soybean powder using 80% methanol-water extractant provided values for total isoflavones extraction with relatively negligible product degradation as the basis for SWE analyses. Preliminary 5 min SWE tests provided optimized conditions as T = 146.23°C, P = 39.8 bar, and α = 20 mg (solid)/mL (extractant). Detailed kinetics modeling suggested the optimum extraction time was 213.5 ± 8.7 min.

The optimum SWE conditions provided a mass transfer of isoflavones promoted by a combination of lowered dielectric constant and diminished fluid density. The development of aglycones rate of formation expressions led to an evaluation of precursor time-concentration profiles and a deeper understanding of the formation and degradation of the components during SWE. Comparison of the yield at the optimized

conditions with previously reported analyses supported SWE as a strongly viable method for isoflavones recovery from okara powders.

CHAPTER 3

Influence of subcritical water process on value addition of soybean byproducts: Correlation and principal component analysis

3.1. Introduction

Antioxidants are known to prevent biological oxidative damage, which is a major cause of various human diseases. Diseases such as cancer, atherosclerosis, diabetes, inflammation, Alzheimer's disease, aging and many others have been linked with biological oxidative cell damage, and the preventive effects of antioxidants on those diseases have been widely investigated [41]. Antioxidants also play a huge role in preventing the degradation of foods by oxidation, which is responsible for rancid flavors and aromas that bring about the unpalatability and unacceptability of foods [42].

There are many sources of antioxidants for human consumption in the form of plants like fruits, vegetables, cereals and legumes. Soybeans are legumes known to contain phenolic compounds such as isoflavones, known for their phytoestrogenic properties, as well as phenolic acids and flavonoids, all of which have been correlated with the antioxidant activity in soybean and soybean-derived products [7], [8]. Various studies have shown that the unconjugated forms of isoflavones (aglycones) are more biologically active and contribute to the antioxidant activity of processed soybean products [9]. Additionally, aglycones are more easily absorbed by humans than their conjugated counterparts (isoflavone glucosides); however, they exist at low

concentrations in unprocessed products (below 5%, compared to over 95 % for conjugated isoflavones) [10, 11].

Different processing techniques increase the concentration of isoflavone aglycones (e.g. fermentation and heat processing) and thus increase the biological activities of the resulting products [43]. One of the methods for human access to soybean antioxidants is through extraction and the addition of the extracts to food as ingredients or the consumption of extracts as supplements. The extraction techniques used conventionally employ the use of organic solvents that are unsafe for human consumption, costly, and environmentally hazardous. They are a big concern for both people and the environment. These drawbacks have led to increased interest in extraction techniques that use solvents and techniques generally recognized as safe (GRAS) [44]. One of the extraction solvents that is safe, green, and readily available in nature is water. It is used in the extraction process near its critical state, known as subcritical water (SW), which is beyond the boiling point and at a high enough pressure to keep water in the liquid state (100–374 °C; 1–220 bar). These conditions bring upon huge changes in water properties. When the temperature of water is fine-tuned to slightly above the boiling point, it becomes an ideal solvent that has an extraction efficiency comparable to or even better than organic solvents. The properties of interest are the decreased dielectric constant similar to organic solvents (e.g. methanol or ethanol), increased viscosity and diffusivity, and decreased water density, all of which make water an ideal solvent for the extraction of bioactive compounds from natural sources [45], [15].

The antioxidant activities of soybean isoflavones have been investigated by Lee, et al., and they found that when genistein and daidzein and their corresponding glucosides were extracted with ethanol, they showed radical scavenging potential, particularly 2,2-

Diphenyl-1-picrylhydrazyl free radicals (DPPH) and Ferric Reducing/ antioxidant power (FRAP), but the antioxidant activities were reported to be ineffective in comparison to tea epicatechins [46]. However, when different extraction techniques were compared, the antioxidant activities of whole yellow and black soybeans were increased during steam processing correlating with the increased total phenolics and isoflavone aglycones [47]. Additionally, Ungar, et al. studied the thermal stability of isoflavone aglycones, genistein and daidzein, their degradation kinetics and the antioxidant activities of their reaction products using 2, 2'-Azinobis (3-ethylbenzothiazoline-6 sulfonic acid) (ABTS) assay [48]. They found a decreased antioxidant potential of genistein due to its thermal degradation.

Subcritical water extraction has the potential to extract bioactive antioxidants from different plant materials [49]. The recovery of bioactive phenolics and isoflavones through this process improves the antioxidant potential of extracts, allowing for the creation of valuable products that could be used as ingredients in foods and nutraceuticals by using only water as the extraction solvent. The aim of this study is to extract Okara phenolic compounds (isoflavones and total phenolics), and to monitor the antioxidant activities (ABTS, DPPH and FRAP) of the resulting extracts. The correlation between isoflavone aglycones and βeta glucosides, as well as total phenolic compounds, with antioxidant activity is studied. This work will help in the understanding of opportunities to add value to byproducts from food processing, particularly soybean byproducts, for the purpose of reducing economic loss while preserving our environment.

3.2. Materials and Methods

3.2.1. Chemicals and Reagents

Isoflavone standards, genistin, daidzin, genistein, and daidzein (HPLC ≥ 99% purity), were obtained from Extrasynthese (Z.I. Lyon Nord, France). Sodium carbonate, anhydrous first grade, was from D.S.P. GR Reagents (256-1, Youbangri, Kyongkido, South Korea), HPLC grade acetonitrile, water, and methanol were obtained from Honeywell (Ulsan, Korea), and acetic acid (99.5% glacial) and hydrochloric acid (1M) were supplied by Samchun (Pyeongtaek, South Korea). Thimbles and syringe filters were from Advantech (Japan). Antioxidant standards (ABTS, DPPH and FRAP) and related reagents were all from Sigma-Aldrich (St. Louis, USA). All other reagents and standards used in this work were obtained from Sigma-Aldrich (St. Louis, USA) of analytical grade, and used without further purification.

3.2.2. Sample preparation

The sample was prepared according to the method in chapter 1.

3.2.3. Subcritical water hydrolysis

Hydrolysis conditions are maintained in the chapter1. The laboratory scale subcritical unit used is described in figure 3.1 below:

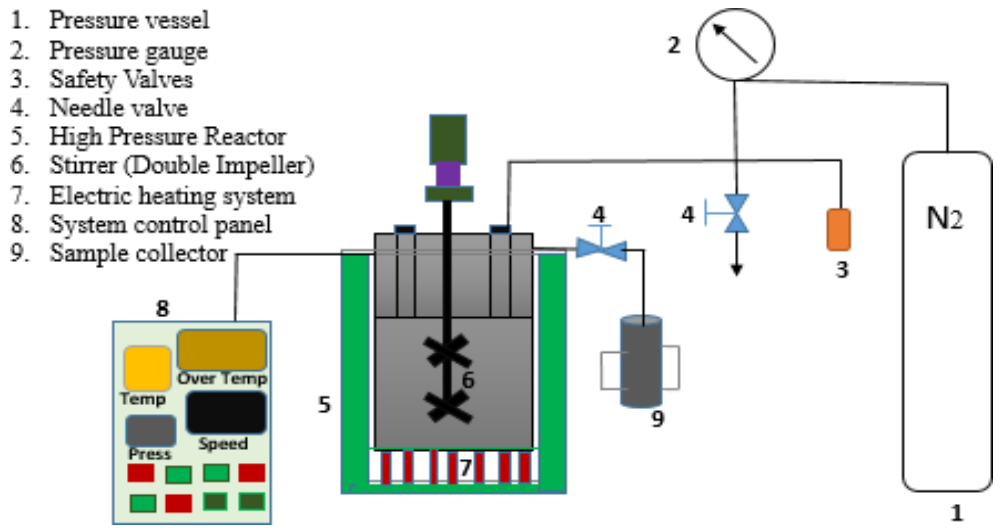


Fig. 3.1. Subcritical water extraction schematic diagram

3.2.4. Total Phenolic Content (TPC) determination

Total phenolic content (TPC) of each soybean by-product hydrolysate was determined using Folin-Ciocalteu assay according to a previous study [50] with modifications. A 1mL aliquot of extracts or standard solution made from 31.25, 62.5, 125, 250, 500 and 1000 mg/L (ppm) dilutions of Gallic acid was added to a 12mL volumetric flask containing 4.5mL of distilled deionized water. Then 0.5mL of Folin-Ciocalteau's phenol reagent was added to the mixture and thoroughly shaken. After 5 minutes, 5mL of Na_2CO_3 solution was added to the mixture. The solution was diluted up to volume with distilled deionized water and mixed well and then Incubated at room temperature in the dark for 90min. Lastly,300 μL of each prepared sample was read at 750nm using a 96-well microplate reader. Total phenolic content of each soybean by-product hydrolysates was expressed as mg gallic acid equivalents per 100g of dry sample (mg GAE/100g). All samples were analyzed in triplicate.

3.2.5. Determination of Antioxidant activity

Antioxidant activity measurement by ABTS assay was determined according to a method by Arnao, *et al* [51] with modifications. Shortly after the formation of ABTS radicals by reacting 7mM ABTS and 2.5mM potassium persulfate, the stable radicals were allowed to react for 12 hours, and then diluted to the absorbance of 0.7±0.02, and the hydrolysates were reacted with the radicals at 1:19 ratio (extracts: radicals), and kept for 2h in the dark prior to reading the absorbance at 734nm with 96 well plate reader. The antioxidant activity of hydrolysates was determined based on the standard calibration curve of Trolox (R^2 = 0.9966) prepared in the concentration range of 12.5 to 100ppm. The scavenging activity of the hydrolysates was determined based on the

decrease in UV absorption and results were expressed as mg of Trolox equivalent per 100g of dry sample (mgTE/100g).

The DPPH assay was conducted according to the method of Cayupán, et al [52] with modifications. The DPPH radical stock solution was prepared by dissolving 72mg of $DPPH^{\bullet}$ in 300mL methanol. The working solution was adjusted by diluting to 0.7±0.02 absorbance at 517nm using a 96-well plate reader. The hydrolysates were allowed to react with DPPH solution at the ratio of 1:19 for 30 min in the dark. The standard calibration curve was linear (R^2 = 0.995) with concentrations ranging from 6.25 to 250 ppm of trolox. Antioxidant activityt was evaluated by measuring the decrease in UV absorption at 517 nm. The results were expressed as (mgTE/100g).

The FRAP assay was performed according to a previous method [53] with modifications. A ferrous tripyridyltriazine complex (Fe^{2+} TPTZ) was formed from Ferric tripyridyltriazine complex Fe^{3+} TPTZ (FRAP) using the stock solutions made of 300 mM acetate buffer (pH 3.6), 10 mM TPTZ solution in 40 mM HCl, and 20 mM $FeCl_3$ $.6H_2O$ solution. Trolox was used as standard antioxidant and the calibration curve was linear (R^2=0.9999) for the concentrations from 16 to 500 ppm. The intense blue color from the formation of $Fe2^{+}$-TPTZ complexes were read at their maximum absorbance of 593nm for both the hydrolysates and standard concentrations. The results were expressed as mgTE/100g fresh mass.

3.2.6. HPLC Analysis

A reverse phase HPLC unit (Hitachi Model-2000 Series, Japan) was used for the analysis of isoflavones according to the method of Wang and Murphy [10] with minor

modifications. Water, acetic acid and acetonitrile were used as mobile phase Separation achieved using an Agilent Eclipse plus C_{18} reverse phase Column.

3.2.7. Statistical analysis

IBM SPSS version 20 software (SPSS.Inc, Chicago, USA) was used to analyze the data statistically. Turkey HSD $p < 0.05$ was used to define the differences within groups, The Pearson correlation coefficient (r) were carried out to define the levels of correlation between variables. The principal component analysis (PCA) was analyzed using MS Excel with XLSTAT software.

3.3. Results and Discussions

3.3.1. Effect of extraction conditions on okara total phenolic contents

The results of the TPC analysis are depicted in fig 3.2. The TPC showed a consistent time - dependent linear increase with a correlation coefficient of $R^2 = 0.936$. The TPC was 68 % of the total content at the initial reaction time of 5 min, and was highest at 245 min extraction time, an increase from 1246 ± 14 and 2090 ± 21 mg GAE/100g respectively. After this time, the TPC began to decrease.

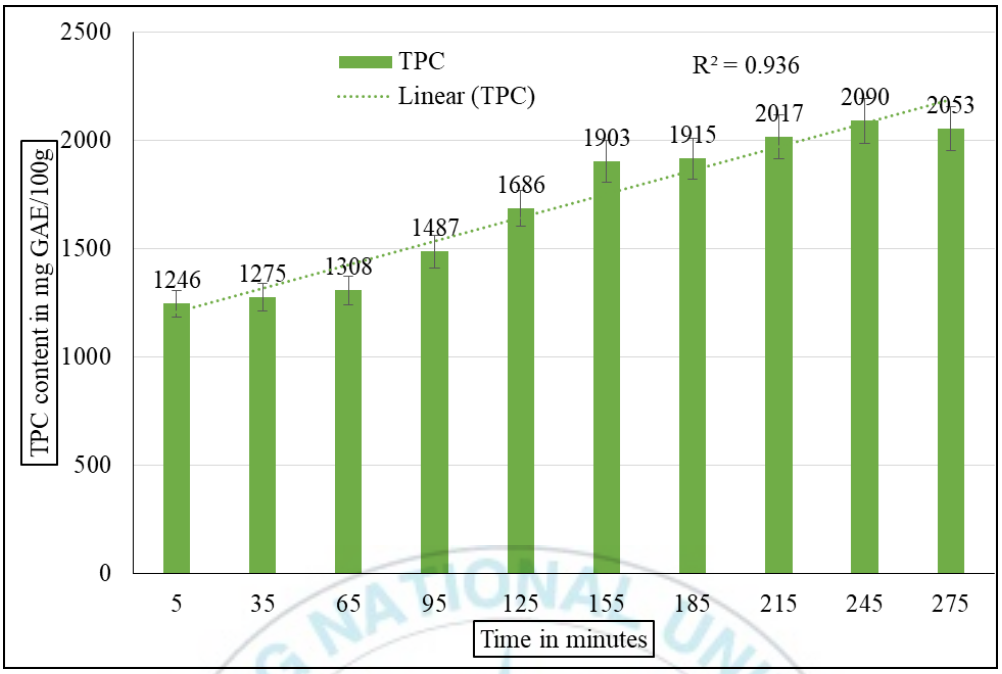


Fig. 3.2 Total Phenolic content of Okara during SWE at different reaction time

The increase of TPC during subcritical water hydrolysis has been reported in previous studies during the extraction of phenolic compounds in different plant products and byproducts. Enhanced recovery of phenolic compounds from *Momordica charantia* (bitter melon) was investigated by Budrat and Shotipruk [54]. They reported an increase of TPC as extraction temperature and time increased when extracting phenolics at different temperatures and reaction times ($130 < T \leq 230$ °C; $10 < \text{Time} \leq 60$ min at 10MPa), the lowest phenolic content at 130 °C while the highest phenolic content was extracted at 150 °C. The latter was consistent with our optimum temperature obtained previously during the optimization of okara isoflavones extraction (146±5 °C) using subcritical water. The increase of reaction time under these conditions increased significantly the TPC. A similar observation was reported by Singh and Saldana who found that when extracting phenolic compounds from potato peels [16], higher amounts of phenolic compounds could be recovered when extraction temperature and time were increased and when subcritical water was used for extraction instead of organic solvents (methanol and ethanol). The time dependent increase of phenolic compounds during subcritical water extraction is due to the increased solubility of phenolics in water as the temperature is increased to around 150 °C, this is driven by the decreased relative static permittivity of water also known as the dielectric constant (ε_r), that decreases as water temperature increases for example the ε_r of water at 25 °C is 78.5 and becomes 55.4 at 100 °C and 1MPa, the higher the temperature increase, the more ε_r decreases to eventually become 34.8 at 200 °C at 1,5MPa. These values correlate to the density parameter values of 0.997, 0.958 and 0.865 g/cm^3 respectively, which are responsible for the elimination of intermolecular interactions between surrounding ions to increase water solvating power[29]. The increase in extraction time under the optimum temperature conditions has shown significant enhancement of the extraction of

phenolics as shown in **Fig. 3.2** The significant increase of TPC could also be a result of the release of phenolics bound to sugar molecules and others in the cell walls due to thermal induced breakdown of covalent and oxygen linkages as well as the cell wall.

3.3.2 Effect of extraction conditions on isoflavones

The effect of subcritical water hydrolysis on the 4 isoflavones studied in this work (Genistein (GE) ,Daidzein (DE) Genistin (GI) and Daidzin (DI)) is shown in **fig. 3.3**. The β-glucoside forms of isoflavones, GI and DI, significantly increased when the reaction time was increased, but they steadly decreased after 35 and 95 min, respectively. These results shows that the isoflavone glucosides have different thermal stabilities in aqueous medium, with DI having higher thermal stability compared to GI . This observation was consistent with the results obtained by Xu et al, [55], who investigated the thermal stability of isoflavones at different temperatures (185 and 215 °C for 3 and 15 min). They observed that daidzin in both conditions, was more stable than genistin. The increase of both genistin and daidzin at 35 and 95 min is caused by their formation through the hydrolytic de-esterification of the malonyl and acetyl forms of the same species. The malonyl forms are well known to be the least stable under heat treatment, and may result in the quick formation of the β-glucoside forms at relatively shorter reaction times. After this period of time, the β-glucosides start to slowly decrease due to hydrolytic degradation, and conversion to their respective aglycones [39].

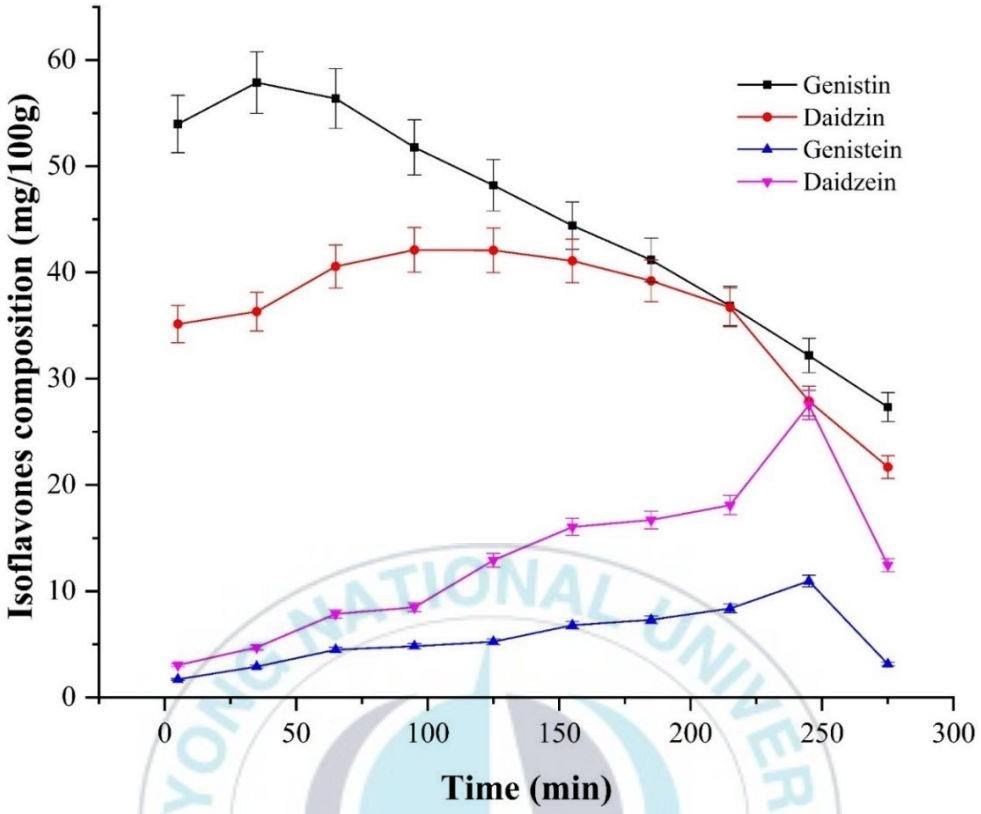


Fig. 3.3. Isoflavones composition of Okara during SWE in different reaction time

Also in Fig.3.3, the concentrations of both isoflavone aglycones were shown to be time – dependent, increase from 5 min to 245 min with 6.5 and 9 fold increases in the concentrations of GE and DE, respectively. The increase of aglycones during heat treatment of whole soybean flour was also investigated by Andrade et al, [40]. They observed changes in isoflavones profiles from oven heating at 100, 150 and 200 °C for 10, 15 and 20 min, with aglycones increasing 3.5 fold after heating at 200 °C for 20 min. Although we similarly found an increase in aglycones, it is possible that the increase from heating at 200 °C for 20 min was limited in comparison to our extraction conditions (146 °C, 40 bar, and 5 to 275 min) due to the higher temperature, resulting in the quick degradation of other isoflavones, preventing the conversion to aglycones. Our extraction conditions applied longer extraction times at a lower temperature, showing a consistent rise in the conversions of conjugated isoflavones to aglycones.

3.3.3 Effect of extraction conditions on antioxidant activities

The antioxidant mechanisms described in Fig 3.4 shows the reactions of unstable radicals and their resulting products after reacting with antioxidants from okara hydrolysates obtained using SWE. The reaction of unstable DPPH* radicals with the source of hydrogen donor result in the formation of stable DPPH shown in the fig.3.4a. Similarly the ABTS and FRAP radicals stabilization in the reactions shown in fig.3.4 b and c are results of increase in antioxidant compounds formed during hydrolysis. Table 3.5 shows the antioxidant activities of okara from each assay. The statistical differences at different reaction times are marked next to the mean values and their respective standard deviations from triplicate samples ($p<0.05$). All antioxidants were significantly

affected by the reaction time as shown in the Table 3.1 for the analysis of variance ($p<0.05$).

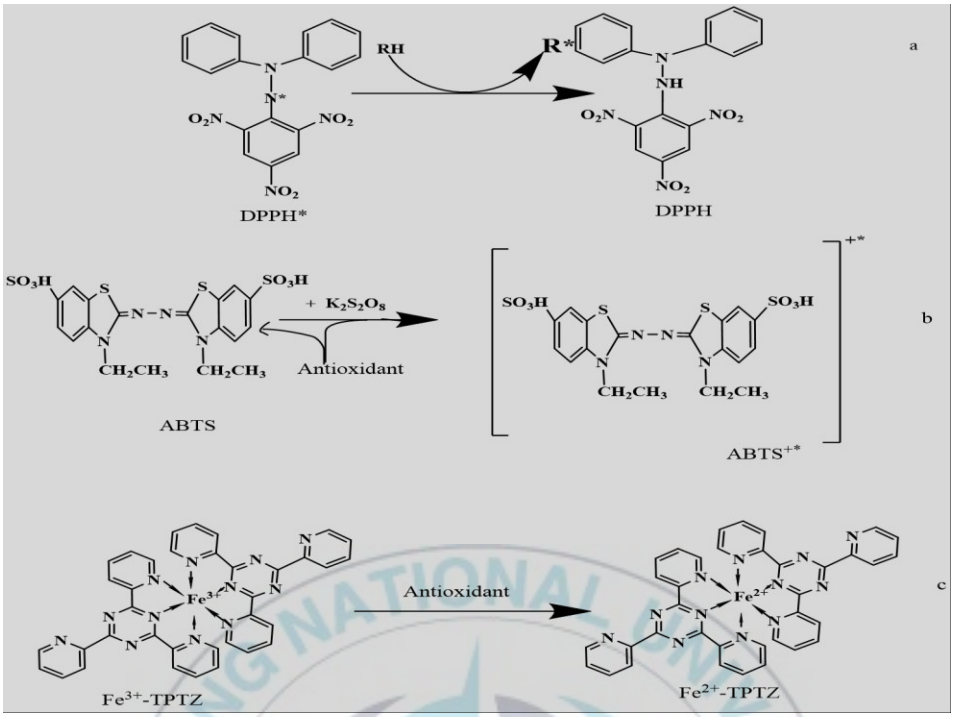


Fig. 3.4. Antioxidant activity reactions mechanisms: **a**: 2, 2- diphenyl-1- picrylhydrazyl (DPPH), **b**: 2, 2'-azino-bis (3-ethylbenzothiazoline -6- sulphonic acid) (ABTS), **c**: Ferric Reducing Antioxidant Power (FRAP).

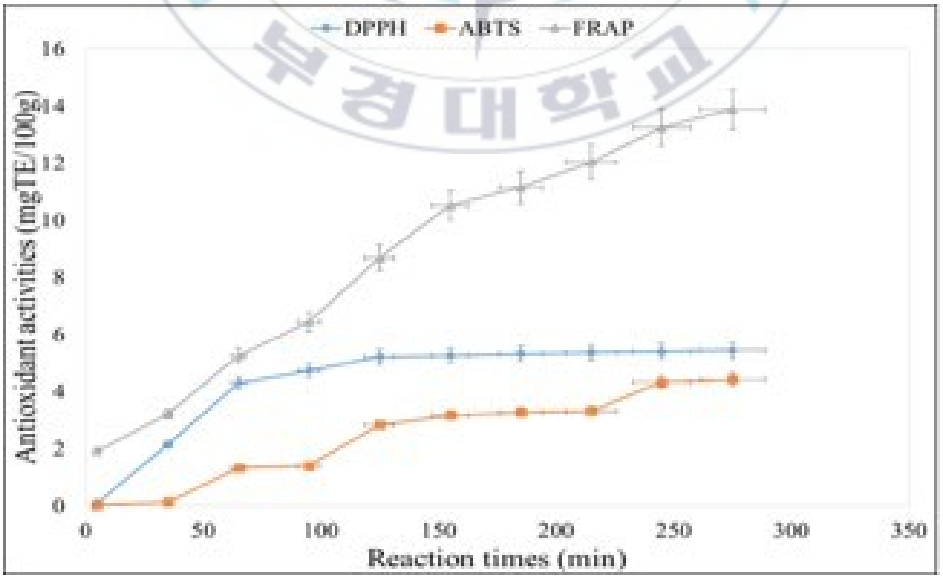


Fig. 3.5. Antioxidant activities (mgTE/100g) of okara at different reaction times. Values are represented as mean and standard deviation ($p<0.05$)

Table 3.1. Anova table of the antioxidants, turkey HSD ($p<0.05$) (SS: Sum of Squares, MS: Mean Square, DF: degree of freedom)

		SS	df	MS	Sig.
ABTS	Between Groups	87.029	9	9.670	.000
	Within Groups	.037	20	.002	
	Total	87.065	29		
DPPH	Between Groups	69.402	9	7.711	.000
	Within Groups	.023	20	.001	
	Total	69.425	29		
FRAP	Between Groups	482.893	9	53.655	.000
	Within Groups	.030	20	.002	
	Total	482.923	29		

ABTS radical scavenging activity of okara extracts increased significantly as the reaction time was increased from 5 to 125 min, rising from 0.10 to 5.22 mgTE/100g, respectively. There was an initial lack of activity shown at 5 min reaction time for DPPH that indicated that the short extraction time was unfavorable for the formation of antioxidants during subcritical water hydrolysis. However, DPPH later consistently increased with longer reaction times from 0.04 mgTE/100g at 5 min up to 2.48 mgTE/100g after 125 min. The FRAP antioxidant activities also increased by extending the reaction time, and were the most sensitive to okara extracts, as shown by the steeper increases. The maximum antioxidant activities for these assays were observed after 275 min with 5.46, 4.44 and 13.88 mgTE/100g for ABTS, DPPH and FRAP assays, respectively. Previously, the extraction of antioxidants from plant wastes and byproducts using subcritical water has been reported. Aliakbarian, *et al*. extracted antioxidants (DPPH) from winery wastes using subcritical water and found it to be more efficient than the organic solvent, ethanol [56]. The effect of subcritical water on the extraction of antioxidants from grape pomace revealed an increased total antioxidant activity; when the temperature was increased, the polyphenol content consequently decreased at temperatures up to 200 °C due to hydrolytic degradation [57]. Although the rise in antioxidant activities during subcritical water extraction has been attributed to the neoformation of antioxidants in natural samples caused by maillard browning, caramelization and thermoxidation reactions at high temperatures up to 200 °C [58], in this study, the increase in antioxidant activities of okara extracts endorse the increase in phenolic contents and isoflavone aglycones .

3.3.4 Correlation analysis

Table 3.2. Shows the correlation between the three antioxidant activity assays with isoflavones and total phenolic contents The Pearson correlation coefficient (r) was used to measure the strength of the correlation between variables.

Table 3.2. Pearson's correlation coefficients between antioxidants, isoflavones and TPC (**Correlation is significant at 0.01 level; *correlation is significant at 0.05 level).

Variables	GI	DI	GE	DE	TPC	ABTS	DPPH
GI	1	0.696**	-0.542**	-0.758**	-0.936**	-0.633**	-0.925**
DI	0.696**	1	0.127	-0.16	-0.419*	-0.133	-0.481**
GE	-0.542**	0.127	1	0.944**	0.719**	0.669**	0.675**
DE	-0.758**	-0.16	0.944**	1	0.871**	0.727**	0.855**
TPC	-0.936**	-0.419*	0.719**	0.871**	1	0.787**	0.972**
ABTS	-0.633**	-0.133	0.669**	0.727**	0.787**	1	0.844**
DPPH	-0.925**	-0.481**	0.675**	0.855**	0.972**	0.844**	1
FRAP	-0.936**	-0.478**	0.691**	0.852**	0.981**	0.851**	0.989**

3.3.5. Correlation between antioxidant activities and TPC

DPPH and FRAP showed very strong correlations with the TPC with r=0.967 and r=0.984, respectively. The correlation, however, was reduced for ABTS with r = 0.781. The strong relationship between antioxidant activity and TPC has been previously observed using different extraction methods, and SWE seems to be more effective for extracting antioxidants. Hassas-Roudsari, *et al*. investigated the antioxidant activity and phenolics extracted from canola meal using SWE and conventional methods, and found that SW showed more efficiency for the extraction of antioxidants (DPPH) and that a correlation existed with total phenolic contents [59]. The beneficial effects of using SW to extract bioactive phenolics and antioxidants in plant wastes and byproducts have also been addressed in a review by Barba, *et al.* [60].

3.3.6. Correlation between antioxidant activities and isoflavones

The correlation between isoflavones and antioxidant activities was investigated as these polyphenols largely contribute to the biological activities of soybeans and soy products. Strong positive and strong negative correlations existed for isoflavone aglycones and conjugated β-glucosides, respectively. GI showed a strong negative correlation to DPPH and FRAP with r= -0.925 and r= -0.936, respectively. A moderately strong negative correlation also existed between GI and ABTS. The correlations between DI and antioxidant activities were weak, and no significant correlation was observed between DI and ABTS. On the contrary, a significant positive correlation was observed with all aglycones and antioxidants. DE exhibited a stronger relationship with antioxidants compared to GE, with both aglycones showing a strong or moderately strong correlation with antioxidants.

The negative correlations between β-glucoside isoflavones (GI and DI) and antioxidants are resultant of the hydrolytic degradations and conversions into aglycones when the reaction time was increased. Likewise, the increase in aglycones (DE and GE) was caused by their conversions from β-glucosides and other forms of isoflavones (loss of glucose groups from malonyl and acetyl glucosides). The isoflavone profile changes during ultra-high temperature processing of soy milk have been described by Zhang, *et al.,* where the stability of aglycones was higher than that of the other forms, and the aglycones increased as a result of conversions from conjugated forms of isoflavones [61]. Subcritical water showed an overall increase in isoflavone aglycones, a decrease in conjugated isoflavones and an increase in antioxidant activities for ABTS, DPPH and FRAP. Our findings were consistent with Xu and Chang, who investigated the effects of different soybean cooking techniques on soybean phenolics, isoflavones and antioxidant activities. They found that pressure steamed soybeans had increased contents of isoflavone aglycones and other phenolics, correlating with increased antioxidant activities (DPPH and FRAP) [62]. In summary, reaction time monitoring during subcritical water extraction under optimum conditions can improve bioactive compounds (isoflavone aglycones and total phenolics), and thus enhance the antioxidant activities of the extracts.

3.3.7 Principal component analysis

Principal component analysis (PCA) was performed to describe the relationship between active variables (reaction time) and active observations (isoflavones, TPC, antioxidant activities) as shown in Fig. 3.6.

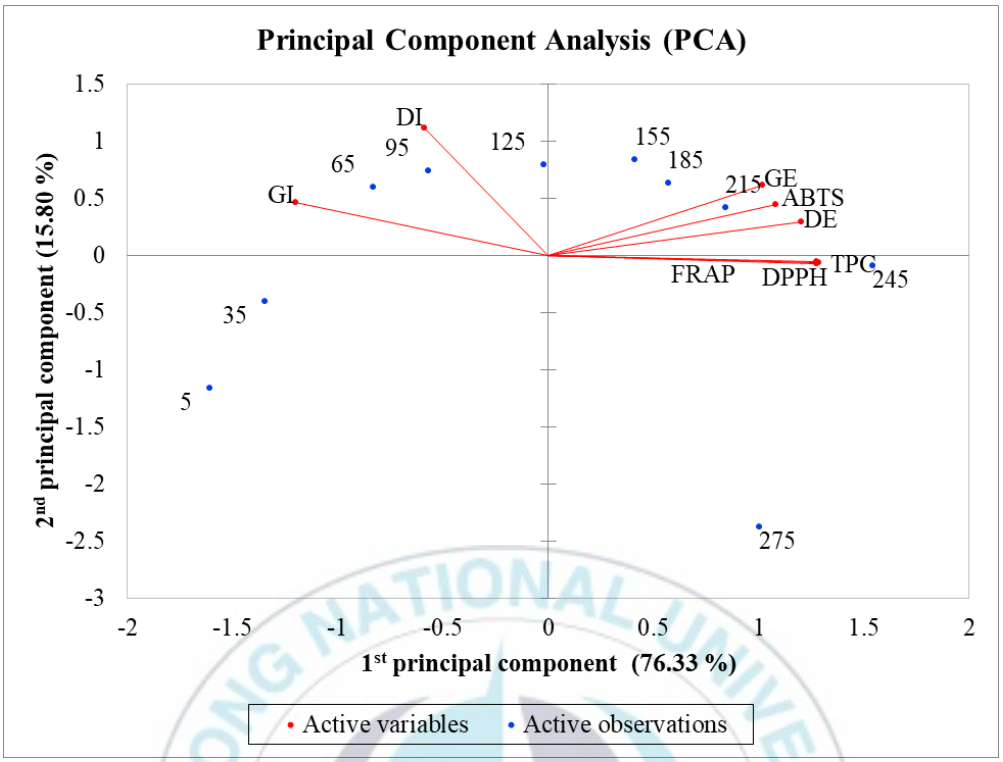


Fig. 3.6. Principal Component Analysis plot of isoflavones (GI, DI, GE and DE), TPC and antioxidant activities (ABTS, DPPH and FRAP) of the okara extracts at different reaction times

This biplot PCA demonstrates the response and correlation of the variables to the increase in reaction time. The observation from the PCA biplot was consistent with the Pearson correlation study in Table 2. The grouping of the vectors, as well as their symmetry, shows the relationship between the variables and the observations. The PCA reached 92.13% of total variance from 15.8 and 76.33% for the 1st and 2nd principal components, respectively. It can be seen that the increased reaction time during SWE grouped the variables GI and DI in the time range between 50 and 110 min. The rest of variables were all clustered in the time range of 210 to 250 min with GE, ABTS and DE clustered, and a negligible angle between FRAP, DPPH and TPC. Altogether, these results indicate a strong positive relationship between isoflavone aglycones and TPC with antioxidant activities, strongly confirming the dependence of the antioxidant effects of okara hydrolysates on the reaction time during SW hydrolysis.

3.4. Conclusion

The potential of the subcritical water extraction of bioactive phenolics and antioxidants from plant byproducts like soybean okara can be thoroughly enhanced by taking the reaction time into consideration. The results obtained during subcritical water extraction with extended reaction times showed that the compositions of bioactive compounds in the hydrolysates were significantly affected by the reaction time. At 245 min, there was a 68 % increase in total phenolic contents from the initial reaction time, representing the highest recovery. Similarly, genistein and daidzein were increased 6.5- and 9-fold, respectively, from 5 to 245 min, while β- glucosides were negatively affected. A significant increase in antioxidant activities was also observed as the reaction time was increased. The strong correlation between antioxidant activities of okara extracts, their total phenolics, and isoflavone contents showed that the extraction time highly affected the activities and the composition of extracts. The use of subcritical water for the extraction of bioactive antioxidants from plants, wastes, and byproducts is economically and environmentally beneficial as long as conditions that ensure efficiency are used. Since exposure of the sample matrix to subcritical conditions may result in undesirable degradation products, the degradation products that are formed during subcritical water extraction and their health effects still need to be identified in future studies.

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Abstract (in Korean)

Characterization and Hydrolytic Kinetics Modeling of Okara Isoflavones Recovered from Subcritical Water Process

아임계 수를 이용하여 추출한 콩 비지 이소플라본의 특성 및 가수분해 속도 모델링

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Abstract

대두를 두유와 두부로 가공 시에 많은 양의 비지가 부산물로 배출된다. 이 것은 탄수화물, 단백질, 지질, 비타민, 미네랄 그리고 이소플라본으로 알려진 생 리 활성 물질과 같은 영양소가 풍부하다. 하지만 이러한 부산물의 높은 수분 함 량과 다량의 유기 화합물의 존재는 폐기시 부패를 일으켜 이산화탄소 발생과 환 경 오염 등의 원인이 된다. 대두의 이소플라본과 그 부산물은 여성의 갱년기 증 상, 뼈 건강 및 유방암을 지연시키는 것 같은 다양한 건강상의 이점과 관련이있 다. 다량 생산되는 부산물의 저활용도 대두 산업에 큰 부담이지만, 생체 활성 성분을 추출하는 기술을 적용함으로써 이소플라본의 저렴한 공급원으로 가치를 평가받을 수 있다.

먼저, 두유와 두부 생산의 부산물인 비지는 최소한의 분해 작용으로 이소플라본을 회수하기 위한 목적으로 아임계 수 추출을 활용하였다. 최적 조건을 찾기 위한 반응 표면 방법론(Response Surface Methodology; RSM)에 따르면 최 적 조건은 T = 146.23°C, P = 39.8 bar, and α=20 mg (solid)/mL (extractant)으로 나 타났다. 각각의 genistein 기반 화합물과 daidzein 기반 화합물에 대한 반응 속도 의 수학적 모델을 각 화합물에 대한 속도 상수 및 시간 의존 농도 프로 파일을 도출하는 일련의 연립 방정식으로 풀었다. 이러한 동역학 분석에 따르면 RSM 최적화 조건에서 최적 추출 시간은 213.5±8.7 min으로 나타났다. 다음으로 아임 계 수를 이용하여 aglycone type 이소플라본, 페놀 성분과 항산화 활성이 뛰어난 제품으로 가공함으로써 경제적 이익을 창출하기 위해 미활용 비지 자원의 가치 를 평가하였다. 이번 연구에서 비지는 중요한 생물 활성 성분들의 회수율을 개 선하기 위해 임계 영역에 가까운 물을 이용한 친환경 기술을 활용하였다. 주요 변수에 대한 추출 조건은 이소플라본 회수 최적화를 위해 이전 연구 (T = 146.23°C, P = 39.8 bar, and α= 20 mg/mL)에서 얻은 최적 조건으로부터 일정하게 유지되었다. 반응 시간은 생물학적으로 이용 가능한 비결합 aglycone type 이소 플라본과 가수분해물의 총 페놀 함량을 증가시키기 위한 핵심 매개 변수로 간 주되었다. 반응 시간은 5분에서 275분까지 유지시켰고, 30분 간격으로 반응물 채취하였다. 2개의 aglycone type 이소플라본인 genistein과 daidzein이 각각 6.5배 와 9배까지 크게 증가하였다. 이러한 결과는 결합된 이소플라본이 aglycone으로 전환되는 것은 시간에 의존함을 확인하였다. 반면, 총 페놀 함량은 245분 후에 최대 68%까지 증가하였다. 가수분해물의 항산화 작용은 TPC, aglycone type 이

소플라본 및 항산화 활성(ABTS, DPPH 및 FRAP)간에 강한 상관 관계를 보여준다. 변수 간의 상관관계를 분석하기 위해 Pearson correlation과 Principal Component Analysis (PCA)를 실시하였다. 이러한 연구 결과는 반응 시간의 증가 가 항산화 활성, aglycone type 이소플라본 및 가수분해물의 총 페놀 함량을 증가 시키는 것을 보여주었다.

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