Extraction, Identification and Antioxidant Activities of Polysaccharides and Phenolic Compounds from Okara Using Subcritical Water Technology

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Abstract

Extracting bioactive compounds, including polysaccharides, total phenolic content, total flavonoid content and so forth, from wild plants has been attracting a great attention in the field of food and drug industry in terms of the potential biological and pharmacological activities. Okara is one of the major byproducts of the tofu and soymilk industry. Recently, subcritical water technology (SWT; 100 °C ≤ T ≤ 374 °C & pressure < 22.1 MPa), a novel sustainable environmental technique, has been applied in the extraction of organic compounds biomass, carbohydrates, and other organic materials, instead of the usage of toxic organic solvent such as methanol and acetone. As an effective empirical and mathematical statistical tool, response surface methodology (RSM) has been applied proverbially to optimize extraction process so as to reduce the number of experimental trials and evaluate the interactions between multiple parameters.

To the best of our knowledge, there are no investigations available regarding the optimum yields and bioactivities of bioactive compounds including polysaccharides (OP), total phenolic content (TPC) and total flavonoid content (TFC), extracted from okara using the subcritical water technology. Therefore, the purpose of the present study was to investigate the highest extraction yields under subcritical water condition variables (temperature, residence time and liquid-solid ratio) using response surface methodology and evaluate the bioactivities including antioxidant activity and anticancer activity. Moreover, the further characteristics and properties of

polysaccharides were analyzed. It can be expected that this study would not only support the adequate and accurate regression model calculated by RSM to extract OP, TPC as well as TFC efficiently, but also provide the theoretical foundation to reuse okara so as to be sustainable to the environment.

1. With single method and response surface methodology, the optimum processing parameters for the crude polysaccharides were determined as follows: temperature 148.3 °C, residence time 11.3 min and liquid-solid ratio 33.0 mL/g, yielding 18.68 ± 0.012% of OP. A wide range of monosaccharide compositions was determined, including rhamnose (4.39%), arabinose (30.28%), galactose (56.67%), glucose (1.41%), xylose (3.40%), mannose (0.34%), glucuronic acid (3.26%) and glucuronic acid (0.24%), identifying OP was a typical heteropolysaccharide fraction. The average molecular value of OP was 236.61 kDa. The infrared spectra showed that the absorption bands of OP were characteristic absorption peaks of polysaccharides. C–H stretching vibrations including CH, CH_2 and CH_3, carbonyl group bending vibration, unsymmetrical carbonyl stretching and C–O stretching were all involved in the sample. IC_{50} values of hydroxyl radical scavenging activity, ABTS scavenging activity and chelating activity of OP were 0.73 mg/mL, 1.49 mg/mL and 1.98 mg/mL, respectively. Moreover, the growth inhibition ratio of human osteosarcoma U2OS was observed by MTT method and when treated OP at the concentration of 2.5 mg/ml, the inhibition ratio was 59.35 ± 1.43%. And

no significant toxicity effect on TIG-3 human normal fibroblast cells was determined (cell viability was 92.23 ± 1.12%).

2. To explore the effects of thermal processing effects on the chemical constituents and antioxidant activity of aqueous extracts obtained from okara using SWT procedure with two other fixed variables (liquid-solid ratio of 30 mL/g and residence time of 10 min) were investigated. The total organic carbon (TOC), total sugar and polysaccharides in extracts were reduced from 30.00 ± 0.73% (180°C) to 20.64 ± 0.73% (230°C), 210.36 ± 4.35 (160°C) to 22.83 ± 0.44 mg GE/g (230°C) and 145.61 ± 3.09 (160°C) to 0.30 ± 0.014 mg GE/g (230°C), respectively. On the contrary, the yields of TPC and TFC enhanced dramatically (23.32 ± 0.14 to 76.18 ± 0.45 mg GAE/g and 15.60 ± 0.87 to 66.12 ± 0.69 mg RE/g, respectively) when temperature increased from 160°C to 220°C. All aqueous extracts exhibited high antioxidant activity, e.g., the highest scavenging activity on DPPH radicals (43.43 ± 0.58 mM AAE/g dry extract) was observed at 220 °C, and the highest scavenging activity ABTS radicals (177.12 ± 8.77 mM AAE/g dry extract) was found at 230 °C. Closed relations of total phenolic content (R^2 = 0.925 for DPPH and R^2 = 0.960 for ABTS) and total flavonoid content (R^2 = 0.945 for DPPH and R^2 = 0.947 for ABTS) of the extracts had closed correlations with the antioxidant activities of the extracts.
3. According to the fact that the enhanced yields of TPC and TFC had been determined using SWT and closed correlations between phenolic compounds

and antioxidant activity were analyzed, single factor method and response surface methodology were carried out in order to optimize the conditions of these two compounds from okara using subcritical water technology. The optimum SWT conditions for TPC as follows: temperature 239.99°C, residence time 5.41 min and liquid-solid ratio 101.36 mL/g, yielding a predicted value of 151.21 mg GAE/g. Also, an estimated yield of TFC was 98.29 mg RE/g under the conditions of temperature 240.63°C, residence time 5.91 min and liquid-solid ratio 101.28 mL/g. The proposed value of DPPH assay was 55.66 mM AAE/g dry extract at the condition of temperature 240.55°C, residence time of 5.54 min and liquid-solid ratio of 100.70 mL/g. According to the Derringer's desirability function approach and the feasibility of apparatus, the optimal conditions were as followed: temperature 240 °C, residence time 5.7 min and liquid-solid ratio 101 mL/g, which predicted the yield of TPC, TFC and DPPH as 151.58 ± 0.35 mg GAE/g, 98.35 ± 0.24 mg RE/g and 56.37 ± 0.13 mM AAE/g dry extract, respectively.

In conclusion, results from this study firstly revealed polysaccharides and phenolic compounds possessing high antioxidant activities could be extracted effectively and efficiently by using SWT and provided the certain operation conditions, laying a solid foundation from pure theories into practice for food and drug industries and making it possible to reutilize okara and other by-product waste.

Contents

List of tables

List of figures

Acronyms and Abbreviations

ABTS	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)
BBD	Box–Behnken design
DC	Dielectric constant
DMEM	Dulbecc’s modified eagle’s medium
DMSO	Dimethyl sulfoxide
DMSO	Dimethyl sulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
EDTA	EthylenediaminetetrAAEetic acid
FBS	Fetal bovine serum
FT-IR	Fourier transform infrared spectroscopy
GAE	Gallic acid equivalent
H_2O_2	Hydrogen peroxide
HO•	Hydroxyl radical
HPAEC	High performance anion exchange chromatography
HWE	Hot-water extraction
KBr	Potassium bromide
Kw	Ionization constant
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
OD	Optical density
OP	Polysaccharides of okara
PAD	Pulsed amperometric detection
PBS	Phosphate buffered saline
PI	Propidium iodide
RE	rutin equivalent
RSM	Response surface methodology
SEM	scanning electronmicroscopy
SWT	Subcritical water technology
TFA	Trifluoroacetic acid
TFC	Total flavonoid content
TOC	total organic carbon
TPC	Total phenolic content
TS	total sugar
Vc	Ascorbic acid

Chapter 1 Introduction

1.1 Soybean and soybean curd residue

Soybean (Glycine max (L) Merrill), one kind of native legumes of China, is well-known for its beneficial properties like high quality proteins (40%) and many other functional substances like phenolic acids and isoflavones (Zhang et al., 2003). As an important cash crop with unique and important traits such as the high content of seed protein and dietary fiber and the ability to perform symbiotic nitrogen fixation, soybean is widely planted in the world and its annual production was 276.41 million tons in 2013 (FAO, 2013). Soybean seed contained various phenolic compounds, and phenolic acids were found to 28 - 72% over total phenol content in soybean seed (Okubo et al., 1992; Seo & Morr, 1984). Generally, soybeans are industrially processed to obtain diverse end products as an important food raw material. Apart from protein isolates, there were many traditional fermented and non-fermented soy foods all over the world. For instance, natto and doenjang (soybean paste) are two kinds of traditional fermented soybean food in Japan and Korean, respectively; soy milk and tofu consumption are steadily increasing in Western countries as the representatives of non-fermented soy foods because of its various bioactive components have been linked to promoting human health such as the prevention of obesity or obesity-related metabolic disorders, the improvement of cardiovascular health and the control of cholesterol (Chung et al., 2011). It is imperative that large quantities of the residue are obtained during the extract fabrication process.

The residue generated as a byproduct from ground soy beans after extraction of the water extractable fraction used to produce soy milk and *tofu*, is called *okara*, draff, *tofukasu*, or *soy pulp*, *dou zha* (Chinese), *bejee* (Korean), and *tempe gembus* (Indonesian). Raw okara, also called soybean curd residue, is a yellowish white material consisting of the insoluble material from soybean seeds which remains in the filter sack when pureed soybeans are filtered for the production of soymilk (O'Toole, 1999).

About 1.1kg of fresh okara is produced from every kilogram of soybeans processed for soy milk (Khare et al., 1993). However, being a mass with around 75% (w.b.) of moisture content, which contains approximately 95% of the soy grain solid components, it can deteriorate rapidly upon exposure to air (Perussello et al., 2012). This problem becomes more prominent in summer when the ambient temperature is rather high with the increasing demand of soy milk (Yang, 2005). Moreover, the highly structured fiber and high water-retention capacity of okara promote a highly viscous product, which makes it difficult to apply, preserve or transport (Nakornpanom et al., 2010). Although a handful of those wastes are consumed as only as feed stuff for domesticated animals or as a fertilizer (Wu et al., 2012), most of them are incinerated like other industrial wastes, which lead to potentially severe environmental problems. For example, combustion will produce a large number of greenhouse gases and high moisture content soy waste would increase the incinerator load (Hsieh & Yang, 2004).

Because of economic and environmental concerns, the utilization of food by-products including okara has been expected to increase and to become more efficient. The Japanese government legislated a Food Recycling Act in 2001, and has promoted the “3Rs” (i.e., reduce, reuse, and recycle) of organic resources generated by the food industries. Numerous food by-products are high in moisture content and are thus often stored by ensiling to avoid the energy costs of drying (Wang & Nishino, 2008). As a

matter of fact, okara contains 25.4 - 28.4% protein, 9.3 - 10.9% oil, 40.2 - 43.6% insoluble fiber, 12.6 - 14.6% soluble fiber and 3.8 - 5.3% soluble carbohydrates on a dry basis (Nakornpanom et al., 2010; Van der Riet et al., 1989). To alleviate problems of waste disposal and increase resource utilization rate, some effective methods have been undertaken to devise uses for this byproduct. Taking fermentation as an example, taking account of the characteristics of rich in water-insoluble ingredients including protein, fat, starch and sugar, fresh okara could be potentially utilized as high quality media for microbial fermentation. The significance of okara in the production of organic acid, enzymes, and single cell protein has been proven in the fermentation industry (Hayashi et al., 1992). Also, several physiological functions of okara have been found, such as anti-oxidation (Mateos-Aparicio et al., 2010), anti-cholesterol action (Matsuo & Hitomi, 1993), and anti-obesity action (Préstamo et al., 2007), which indicates that it can be recognized as an important functional food material with potential use in the food industry.

1.2 Subcritical water technology

1.2.1 Basic concept

Extraction is the first stage in the isolation of bioactive compounds from agro-industrial residues and plant materials (Azmir et al., 2013). Since bioactive compounds in bio-resources are typically present in low concentrations, it is of importance to develop more effective and selective extraction methods for the recovery of the desired ingredients. At present, various techniques have been applied to recover targeted compounds from natural sources including hot water extraction (HWE), solid-liquid extraction with organic solvents, ultrasound-assisted extraction, microwave assisted

extraction, supercritical fluids extraction, and high pressure processes (Liau et al., 2010; Tsubaki et al., 2016; Zhao et al., 2016). However, some of the aforementioned methods have some inevitable drawbacks. For instance, HWE, the primary industrial extraction method, requires long extraction time and constant stirring to obtain high efficiencies (Chao et al., 2013). While the traditional organic solvent-based extraction is widely employed for phenolics extraction from vegetable sources, residual toxic organic solvents in extracts can deteriorate the quality of the extracts and can cause serious health problems when the extracts are taken into the human body. Besides, other methods like ultrasound-assisted extraction and microwave assisted extraction often suffers from low extraction yields, long extraction times and sophisticated treatment parameters (Kim et al., 2009).

Recently, subcritical water technology (SWT) has become an interesting alternative in the extensive research activities about the conversion of biomass, carbohydrates and other organic materials in the production of useful chemical compounds and energy. It is a novel extraction for both polar and non-polar compounds based on the region of condensed phase of water between the temperature range from 100^{o}C (boiling point of water) to 374 °C (critical point of water) and a pressure that is sufficiently high to maintain the liquid state (>40 atm, or 4×10^6 Pa) (Anekpankul et al., 2007). Other common terms such as “pressurized hot water”, “superheated water”, “near critical water”, “subcritical water”, “high temperature extraction” and “extraction using hot compressed water” have also been used (Teo et al., 2010). Under the subcritical condition, water maintained in the liquid state during the whole extraction

procedure has unique characteristics such as high density, high reactivity, good solubility for a series of hydrophobic organic compounds having relatively low molecular weights and the ability to hydrolyze ester and ether bonds contained in polymer chains (Baek et al., 2008). Subcritical water has been gaining increasing attention as an environmentally friendly solvent and attractive reaction medium for a variety of applications. Unlike conventional extraction technologies, SWT presents a series of important advantages like cheapness, non-toxicity, non-flammability, non-explosion and offers essential benefits compared to other substances, particularly in the field of “green chemistry”. Using water as the reactant in the media gets over some of the disadvantages of traditional methods. One of the most significant advantages of using subcritical water on biomass samples is that no pretreatment of wastes (such as drying and crashing) is required (Asghari & Yoshida, 2010). Although subcritical water method requires electricity to maintain the high temperature and high pressure, the power consumption can be neglected under the situations such as massive processing and steady states.

1.2.2 Reaction mechanisms

The change of dielectric constant (ε, as a measure of its polarity) and ionization constant contributes to the reactivity of subcritical water with certain hydrophobic organic compounds. In particular, as the temperature increases, the hydrogen bond between water molecules weakening induces the decrease of dielectric constant, which in turn changes water become non-polarity, with the result that water has been proposed

as an alternative to the use of relatively non-polar organic solvents for some applications (Ndlela et al., 2012). For example, although the value of dielectric constant of water at room temperature is nearly 80, it can be decreased to nearly 27 at 250ºC, being at these conditions a similar value to that of some organic solvents like ethanol or methanol at ambient temperature. In other words, the similarity of water under subcritical conditions is sufficient to dissolve extract polar, moderately polar and non-polar substances. As for ionization constant, the ion product of water (*K*w) of the subcritical water substantially as the temperature rises to around 270 ◦C (Marshall & Franck, 1981) and subcritical water can catalyze chemical reactions such as hydrolysis and degradation without any additional catalyst. Generally, the positive and negative ion concentrations keep balance, whereas the thermal energy makes hydrogen bond fractures, thereby breaking the balance and inducing an increase in the ion product of the liquid during the extraction procedure. Additionally, other physicochemical characteristics like the density, dissociation constant, viscosity, diffusivity and solvency of the water all change, allowing for the dissolution and reaction of organic materials: the high density enhance the solvency of the water (Ndlela et al., 2012); the dissociation constant of water at temperatures of 200-300ºC is three orders of magnitude greater than that of ambient water (Asghari & Yoshida, 2010); the reduce viscosity of the water allows a better penetration of the solvent in the matrix, consequently favoring the extraction rate (Ong et al., 2006); the presence of water at high temperatures as a reactant accelerates hydrolysis reactions and facilitates the conversion of biomass to sugars.

From a kinetics standpoint, four sequential steps taking place in the extraction system filled with sample materials under elevated temperature and the pressurized conditions control the efficiency of the extraction procedure (Teo et al., 2010), including:

(1) Desorption of solutes from the various active sites in the sample matrix to bound targets;

(2) Diffusion of subcritical water into the organic matrix;

(3) Dissolution of the compounds from original binding sites into the solvent.

(4) Elution of the extract-laden solution from the sample matrix into the collection chamber.

Based on these features, subcritical water can be used as an extractant instead of organic liquid solvent extraction techniques for its efficiency, environmentally friendly characteristics and high catalytic activity.

1.2.3 Relevant researches

In recent years, several attempts at extracting valuable materials using SWT from herbal plants and foodstuffs have been reported. It is well-known that plants are essential sources for flavors and fragrances and many papers have been published on the applicability of SWT for the extraction. For instance, SWT has been employed to extract anthocyanins from Brassica oleracea (Arapitsas & Turner, 2008), anthraquinones from Morinda citrifolia (Kiathevest et al., 2009), glycyrrhizin from Glycyrrhiza glabra (Mukhopadhyay & Panja, 2008), Lignans from Linum

usitatissimum (Cacace & Mazza, 2006), essential oils from Origanum micrathum (Gogus et al., 2005), Tanshinone I and IIA from Salvia miltiorrhiza (Ong & Len, 2004), as well as Shikimic acid from Chinese star anise (Illicium verum Hook. f.) (Ohira et al., 2009). Furthermore, methods using SWT have been applied successfully on food materials for the extraction of their bioactive compounds. It has been shown that total sugars, proteins from defatted rice bran could be extracted with subcritical water at 200^{o}C for 5 min (Hata et al., 2008), isoflavones from defatted soybean flakes could be extracted with subcritical water at 641 psig and 110^{o}C for 2.3h (Li-Hsun et al., 2004). SWT has recently been used to extract the phenolic compounds from potato peel (Singh & Saldaña, 2011), catechins and proanthocyanidins from winery by-products (García-Marino et al., 2006), dietary fiber from *Citrus junos* peel (Tanaka et al., 2012), ginsenosides from American ginseng (Choi et al., 2003). Some carbohydrate compounds were extracted using SWT in the past years like monosaccharides from seaweed (Meillisa et al., 2015), polysaccharides from *Lycium barbarum L* (Chao et al., 2013).

1.3 Polysaccharides

Polysaccharides are an important class of polymeric carbohydrate molecules composed of long chains of monosaccharide units joined together by glycosidic linkages, usually found in almost all living organisms such as seaweeds (alginate, agar-agar and carrageenan) (Fernández et al., 2015), plants (cellulose, hemicelluloses, pectin and guar gum) (Roversi & Piazza, 2016), microorganisms (dextran and xanthan gum) (Yoo et al., 2004), and animals (hyaluronan, chondroitin, chitin and heparin) (Sinha &

Kumria, 2001). Polysaccharides have a general formula of $C_x(H_2O)_y$ where x is usually a large number between 200 and 2500. Given the fact that the repeating units in the polymer backbone are often six-carbon monosaccharides, the general formula can also be represented as $(C_6H_{10}O_5)n$ where $40 \leq n \leq 3000$ (Zong et al., 2012). Polysaccharides can be classified in various possible ways, such as on the basis of structure, chemical composition, solubility, sources, and applications. Taking sources as an example, these obtained from various organisms, such as algae, plants, microorganisms, and animals are defined as natural polysaccharides; in contrast, semi-synthetic polysaccharides are produced through the chemical or enzymatic modification methods (Polysaccharide-based anticancer pro-drugs).

Polysaccharides play diverse and pivotal roles in many biological processes. Apart from energy storage (e.g. starch and glycogen) and structural components (e.g. cellulose in plants cell walls and exoskeletons of crustaceans) (Klemm et al., 2005; Pillai et al., 2009), polysaccharides and their derivatives participate in signal recognition and cell-cell communication and also play key roles in the immune system, fertilization, pathogenesis prevention, blood clotting, and system development (Naveen Chandra et al., 2011)(Zong et al., 2012). Additionally, it has been demonstrated that polysaccharides have a broad spectrum of biological effects, such as antibiotic, antioxidant, anti-mutant, anticoagulant, and immune-stimulation activities. Therefore, polysaccharides together with polynucleotides, proteins, and lipids constitute the most important four bio-macromolecules in life science (Liu et al., 2015).

Polysaccharides are widespread in nature, and account for an estimated 66% of all global bound carbon (Gruber, 1976). In spite of bioactive properties and sufficient content, some issues like weak solubility, dispersion in various media and unsuitable hydrophilic/hydrophobic balance make it inconvenient to utilize polysaccharides in their native forms, especially truth methods of extraction can affect their chemical compositions and structures (Li et al., 2008).

1.4 Phenolic compounds

Being a class of chemical compounds consisting of a hydroxyl group (-OH) bonded directly to an aromatic hydrocarbon group, phenolic content, sometimes called phenolics, are reported to more than 8000 in various vegetables, fruits, cereal crops, etc. (Buchanan et al., 2015). Phenolics are responsible for structural and protective functions in plants, contributing to flavor, color, astringency and bitterness of fruits and vegetables (Pandey & Rizvi, 2009).

Phenolics are classified as simple phenols (phenolic acids and coumarins) and polyphenols (flavonoids, stilbenes, lignans and tannins) based on the number of phenol units in the molecule (Khoddami et al., 2013). Among these compounds, flavonoids constitute the largest group of plant phenolics, accounting for over half of the eight thousand naturally occurring phenolics (Baxter et al., 2009). Besides, Variations in substitution patterns to ring C in the structure of these compounds result in the major flavonoid classes, which include flavonols (e.g., quercetin and kaempferol, the most ubiquitous flavonoids in foods), flavones, isoflavones, flavanones, anthocyanidins (pigments responsible for the colour of most fruits), flavanols (catechins-monomers and proanthocyanidins-polymers, known as condensed tannins) (Petti & Scully, 2009).

From the perspective of pharmacology, phenolics have received considerable attention due to their protective actions against chronic degenerative diseases like cataracts, macular degeneration, neurodegenerative diseases, and diabetes mellitus (Chung et al., 2011). Moreover, through in vitro or in vivo experiments, phenolics have been studied for minimizing or even eliminating an initiation or progress of various human diseases (Zand et al., 2002). It should be pointed that Phenolic antioxidants such as phenolic acids may modulate cellular oxidative status and prevent biologically important molecules such as DNA, proteins, and membrane lipids from oxidative damage (Yu et al., 2002). For their bioactive properties, phenolics have been becoming an increased demand to produce “functional” or “nutraceutical” foods (Soto et al., 2011).

1.5 Targets and structure of the thesis

Re-utilization of agro-industrial residue like okara has been increasingly attracting the attention of researchers, considering its potential bioactive compounds. There is no doubt that excessive consumption of non-renewable resources puts human beings in a dilemma about predictable resource depletion in the near future. Being a large quantity of sustainable agriculture resource waste, okara has been proved to possess affluent active ingredients including polysaccharides, phenolic compounds, and so forth, which can be further extracted as antioxidant materials for food and drug industries. In order to recover such desired ingredients more effectively and selectively from okara, an alternatively novel extraction technology called SWT was employed to extract polysaccharides and phenolic compounds (TPC and TFC) from okara. Therefore, the objective of this research work was to obtain the optimum extraction conditions of

polysaccharides and TPC and TFC using subcritical water technology, identify the composition of polysaccharides and evaluate their antioxidant activities *in vitro*. Specifically, this thesis consisted of five chapters in which the major points were listed as follows:

Chapter 1 Introduction

In this chapter, the basic information including soybean, okara was introduced; the definition, mechanism and relevant studies of subcritical water technology were given; three kinds of target compounds (polysaccharides, TPC and TFC) were addressed. Also, the targets and the structure of the thesis were indicated.

Chapter 2 Enhanced production, identification and antioxidant activity of polysaccharides from okara by using subcritical water technology

In this chapter, polysaccharides from okara using SWT were extracted and identified. Antioxidant capacities in vitro and cell assays were also assayed

Chapter 3 Thermal processing effects on the chemical constituents and antioxidant activity of extracts from okara

In this chapter, okara extracts treated by different elevated temperatures was conducted to detect the thermal processing under subcritical water conditions effects on the change of specific constituents and their antioxidant activities.

Chapter 4 Optimization of subcritical water extraction of phenolic compounds from okara using response surface methodology

In this chapter, the optimum conditions of TPC, TFC and IC_{50} value on DPPH radicals of extracts using by SWT from okara were analyzed by single factor and

response surface methodology, considering these variables including temperature, residence time and liquid-solid ratio.

Chapter 5 Conclusions and future researches

The previous researches were compendiously concluded and future projects were prospected.

Chapter 2 Enhanced production, identification and antioxidant activity of polysaccharides from okara by using subcritical water technology

2.1 Introduction

Due to its characteristics of high moisture content and short shelf life, most okara is disposed as agro-industrial waste or treated by incineration, although sometimes it is utilized as feedstuff for domestic animals. However, okara is rich in active ingredients like polysaccharides, which have been proved to obtain potential biological and pharmacological activities. Therefore, it is become extremely important and necessary to extract polysaccharides from okara, especially in food and drug industrial production.

Conventional extraction methods, such as hot water extraction (HWE), organic solvent extraction, and enzyme hydrolysis, have been widely employed (Li et al., 2013; Mateos-Aparicio et al., 2010; Villanueva-Suárez et al., 2013). However, these methods have some drawbacks including inefficiency (long reaction time and low extraction yield), large consumption of toxic and flammable organic solvents and rigorous enzymatic depolymerisation conditions. Recently, subcritical water technology has been subjected to extract polysaccharides from natural resources (Chao et al., 2013; Liu et al., 2016; Wang & Lü, 2014). It has been acknowledged that SWT using water as the solvent can produce high yields within a short reaction time for a number of hydrophobic organic compounds (Herrero et al., 2006). Previous works indicate that SWT is an efficient process to produce polysaccharides from agricultural biomass,

however, currently little information can be found on polysaccharides production from okara (OP) and its compositions. In addition, since SWT is a multi-parameter dependent and complicated process, optimization of the operation conditions is the first step for future application of this technology for polysaccharides production in practice. Furthermore, antioxidant activities of OP obtained by using SWT are also unknown.

The present study was designed by response surface methodology (RSM) to achieve the optimal conditions like temperature, residence time and liquid-solid ratio for yielding the maximum okara polysaccharides (OP). In addition to analysis and identification of OP composition, *in vitro* antioxidant activity of the obtained OP was also evaluated.

2.2 Materials and methods

2.2.1 Chemicals and reagents

Ascorbic acid, hydrogen peroxide, sodium hydroxide, sodium acetate trihydrate, chloride ferric, ferrous sulphate, dibasic sodium phosphate, sodium dihydrogen phosphate, sodium salicylate, ethanol, chloroform, n-butano, potassium persulphate, phenol and D-glucose were purchased from Wako Pure Chemical (Osaka, Japan). Trifluoroacetic acid, monosaccharide standards and 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) were purchased from Sigma-Aldrich, Inc. (Saint Louis, MO, USA). All the chemical reagents were of analytical grade.

2.2.2 Pretreatment of okara sample

Okara with moisture content of 78.8% was purchased from Inamoto Co., Ltd. (Tsukuba, Japan). Residual water was removed by drying at 60°C for 5 h. The dried okara was ground in a high speed disintegrator (IFM-800, IWATANI, JAPAN) to obtain a fine powder (250 μm) and then refluxed with 85% ethanol at 70°C for 4 h to defat, deactivate enzymes and remove interference components. The residue was thermally dried at 50°C and vacuum packed to decrease lipid oxidation.

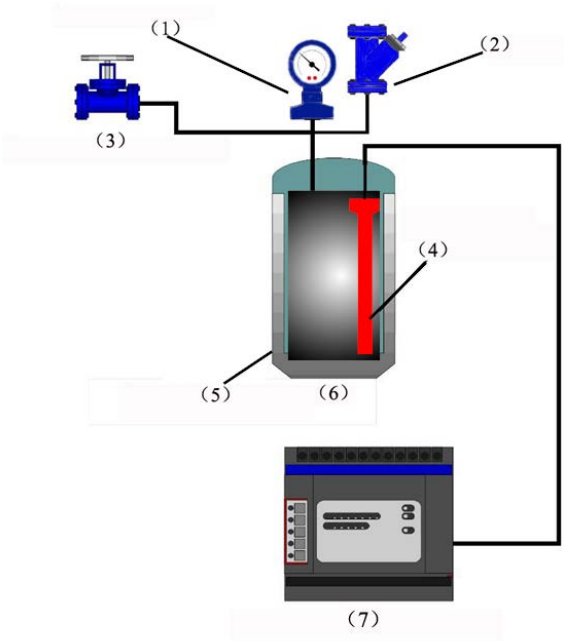


Fig. 2-1 Experimental schematic diagram of subcritical water system for crude polysaccharides production from okara. (1) Pressure gauge, (2) Safety head, (3) Pressure release valve, (4) Temperature sensor, (5) Insulation jacket, (6) SWT reactor, (7) Temperature controller and thermocouple.

2.2.3 Optimization of SWT process for OP production by using RSM

In this study, okara was obtained from the same batch in the manufacturing process. Pretreated okara (1.0g) was treated by SWT under the designed temperature, residence

time and liquid-solid ratio in the reactor system (MMS-200, OMLABO, JAPAN), as shown in Fig. 2-1. After SWT treatment, the supernatant was separated from the solid residue by centrifugation (7500 rpm at 4°C for 15 min) followed by filtration. Then the filtrate was precipitated by adding dehydrated ethanol to a final concentration of 80% (v/v) and stored at 4°C for 12 h. The precipitate was harvested as crude OP after centrifugation at 7500 rpm for 15 min, washed triple times using dehydrated ethanol. After being re-dissolved in ultrapure water, the aqueous solution was subjected to remove proteins by using Sevag reagent (chloroform and n-butanol in a 4:1 ratio), dialyzed with deionized water for 72 h, concentrated under reduced pressure. Finally, the OP product was collected after lyophilization. OP yield (%) was calculated according to Eq. (2-1).

$$\text{OP yield (\%)} = W_{OP}/W_{okara} \times 100\% \quad (2\text{-}1)$$

where W_{OP} is the dry weight of OP obtained, and W_{okara} is the dry weight of okara sample used for SWT treatment. All experiments were performed in triplicate.

In the optimization of SWT operation conditions, response surface methodology (RSM) was employed due to its capability of a large reduction in the number of experimental trials and evaluation of interactions between multiple parameters (Wang et al., 2012). Box-Behnken design (BBD) is more apt to arrange and interpret experimental results through fitting a second-order polynomial by a least squares technique. After our preliminary trials by a single factor test (Fig. 2-2), SWT treatment experiments with the three independent variables (X_1, temperature; X_2, residence time; and X_3, liquid-solid ratio) at three levels (Table 2-1) were performed to statistically

optimize OP production by using BBD method. For statistical calculation, the variables were coded according to the following equation Eq. (2-2):

$$x_i = (X_i - X_0)/\Delta X \quad i = 1, 2, 3 \tag{2-2}$$

where x_i is the coded value of the independent variable, X_i is the actual value of the independent variable, X_0 is the actual value of X_i at the central point, and ΔX is the step change value.

Table 2-1 Independent variables and their levels used in the response surface design (BBD).

Independent variables	Symbol	Levels		
		-1	0	1
Temperature (°C)	X_1	130	150	170
Residence time (min)	X_2	5	10	15
Liquid-solid ratio (mL/g)	X_3	20	30	40

In order to predict the optimal conditions, experimental data were analyzed using Design-Expert software (v.8.0.5 trial, Stat-Ease, Inc., Minneapolis, USA) and fitted to the following quadratic polynomial model (Eq. (2-3)):

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j \tag{2-3}$$

where Y is the predicted response variable associated with each three level combination (OP yield), X_i and X_j are the levels of the independently coded variables ($i \neq j$) affecting the response of Y, B_0, B_i, B_{ii} and B_{ij} are the regression coefficients for intercept, linear, quadratic and interaction terms, respectively. The model can be used to evaluate the effects of each independent variable on the response. Analysis of the experimental design and calculation of predicted data was conducted using Design-Expert software to estimate the response of the independent variables.

2.2.4 Characterization of OP

The composition of monosaccharides in yielded OP was firstly determined by using high performance anion exchange chromatography (HPAEC) with pulsed amperometric detection (PAD) as described previously (Cai et al., 2016) with some modifications. Briefly, approximately 10 mg of lyophilized polysaccharides sample extracted under the optimum conditions was hydrolyzed in 4 mL of 4 mol/L trifluoroacetic acid (TFA). After incubation at 120°C under nitrogen for 4 h and then cooling to ambient temperature, the hydrolyzate was centrifuged and evaporated to remove the rest of TFA. The resulted solution, after being added with ultrapure water to 5 mL, was diluted for 50 times. Being filtered through 0.2 μm pore membrane filter, the derivatives were isolated on a Dionex ICS-3000 system (Dionex, Sunnyvale, CA, USA) with an advanced gradient pump and an eluent degas module. Chromatographic column used in this study was a Carbo PAC™ PA10 column (4 × 250 mm). 10 μL of the resultant solution was employed to elute at a flow rate of 1 mL/min at a constant temperature of 35°C to get the ion chromatographs. Various concentrations of NaOH were tested in this work. H_2O (eluent A), 200 mM NaOH (eluent B) and 1 M CH_3COONa (eluent C) were used as the mobile phase. A gradient elution of 0–100% by mobile phase A to C for 0-20-20 was performed for 1-35 min. The elution program was as follows: 0–20 min (91% A, 9% B and 0% C), 20–20.1 min (86% A, 9% B and 5% C), 20.1–35 min (71% A, 9% B and 20% C). The retention time of each monosaccharide standard in the mixtures under different elution conditions was confirmed by the analysis of the corresponding monosaccharide.

The molecular weight of OP was measured by the method described by (Yang et al., 2016) with some modifications. A high performance size elusion chromatography coupled with multi-angle static light scattering with and refractive index (HPSEC-MALLS-RID) system was used. The HPSEC-MALLS-RID system consists of a pump (e2695,Waters, USA), a HPSEC columns (TSKgel SuperMultipore PW-M,TOSOH, Japan), a MALLS detector (DAWN HELEOSII, Wyatt Technology, Santa Barbara, CA, USA), and a RI detector (OPtilab T-rex, Wyatt Technology, Santa Barbara, CA, USA). Other parameters are as follows: collection Astra Version is 5.3.4.20; cell type is fused silica; laser wavelength is 658.0 nm; calibration constant is 2.9267e-5 1/(V cm); UV Instrument is Generic UV instrument (280 nm) and Refractive index is 1.331. Mw, Mw/Mn and Rg of the polysaccharide fraction were determined under light scattering intensity of different angles at 25°C. OP (10 mg/mL) was filtered on a 0.45 μm pore membrane before injection (20 μL) and eluted with water (0.5 mL/min).

FT-IR was also used to identify the obtained OP, which was carried out with the KBr-disk method (Li & Shah, 2014) using a Jasco FTIR 3000 spectrometer (Jasco, Wakayama, Japan). The dried OP was mixed thoroughly with potassium bromide (KBr) powder, ground and pressed into 1-mm pellet for spectrometric measurement at a frequency range of 400 – 4000 cm^{-1}.

2.2.5 Antioxidant activities of polysaccharides

In this study, antioxidant activities were assessed by ABTS radicals and hydroxyl radicals scavenging activities, according to the methods used by Re et al. (1999) and Ge et al. (2014) with some modifications, respectively.

Constant volume (0.15 mL) of various concentrations of samples (0.3125-10.00 mg/mL) was reacted with 2.85 mL of $ABTS^{\cdot+}$ solution after being mixed vigorously. The absorbance was measured at 734 nm after incubation at ambient temperature for 10 min. Ascorbic acid was used as the positive control. The scavenging activity of ABTS free radicals was calculated according to the following equation Eq. (2-4):

$$\text{Scavenging activity (\%)} = [1 - (A_1 - A_2)/(A_3 - A_4)] \times 100\% \quad (2\text{-}4)$$

where A_1 is the absorbance of (ABTS solution + sample), A_2 is the absorbance of (potassium persulphate + sample), A_3 is the absorbance of (ABTS solution + distilled water) and A_4 is the absorbance of (potassium persulphate + distilled water).

Hydroxyl free radicals generated from $FeSO_4$ and H_2O_2 were detected by their ability to hydroxylate salicylate. The reaction mixture (2.5 mL) contained 0.5 mL of $FeSO_4$ (1.5 mM), 0.35 mL of H_2O_2 (6 mM), 0.15 mL of sodium salicylate (20 mM), and 1.0 mL of different concentrations of the OP. Ascorbic acid was used as the positive control. The absorbance of the hydroxylated salicylate complex was measured at 562 nm after incubation at 37°C for 1 h. The scavenging effect on hydroxyl free radicals was calculated as Eq. (2-5).

$$\text{Scavenging activity (\%)} = [1 - (A_1 - A_2)/A_0] \times 100\% \quad (2\text{-}5)$$

where A_1 is the absorbance of the sample or ascorbic acid, and A_0 is the absorbance of the solvent control, whereas A_2 is the absorbance of the reagent blank without sodium salicylate.

2.2.6 Comparison between SWT and HWE

Being as the control, hot water extraction (HWE) was also conducted to obtain OP from okara. Comparison was performed between the yields, SEM image and antioxidant activities of OP by using SWT and HWE in this work. As for HWE process,

restated, pretreated dry powder (1.0 g) was extracted for 120 min at 30 mL/g (water to okara ratio) by using boiling water.

2.2.7 Cell assays

Human normal fibroblast cells (TIG-3) and Human osteosarcoma cell lines (U2OS) obtained from Japanese Collection of Research Bioresources (JCRB) Cell Bank were employed to . Both cells were maintained in Minimum Essential Medium Eagle medium (Sigma Chemical Co., Saint Louis, MO) containing 10% (v/v) fetal bovine serum and antibiotics (consisting of 100 U/ml penicillin and 100 μg/ml streptomycin) at 37°C in 5% (v/v) CO_2 atmosphere. Cells were cultured for 2-3 days to reach the logarithmic phase and used for experiment.

TIG-3 and U2OS were grown in DMEM medium at 37°C in a 5 % CO_2 atmosphere to logarithmic phase. Cells were harvested, and an aliquot (100 μl) of cell suspension (5×10^4 cells/mL) were dispensed into a 96-well plate (2×10^3 cells/well) and pre-incubated at 37°C in a 5 % CO_2 atmosphere for 24 h. Then cells were exposed to various concentrations (0.15625-2.5 mg/mL) of OP for 72 h. After drugs exposure, 96-well plates were removed from incubator and 20μl MTT stock solution was added to each well incubated at 37°C, 5% CO_2 for 4 hours. Afterwards, 96-well plates were removed from incubator and aspirated the solution and further added 100 μl DMSO to each well and rotated the plate for 5 min to distribute evenly. Ultimately, absorbance was measured with an ELISA reader at 540 nm.

2.2.8 Statistical analysis

Analysis of the experimental design and data was performed using Design-Expert software of version 8.0.5 (Stat-Ease Inc., Min-neapolis, USA). All experimental results were expressed as means ± SD of triplicate experiments. All analyses were performed

using the Statistical Package for the Social Sciences (SPSS, 19.0). Statistically significant was assumed at $P < 0.05$.

2.3 Results and discussion

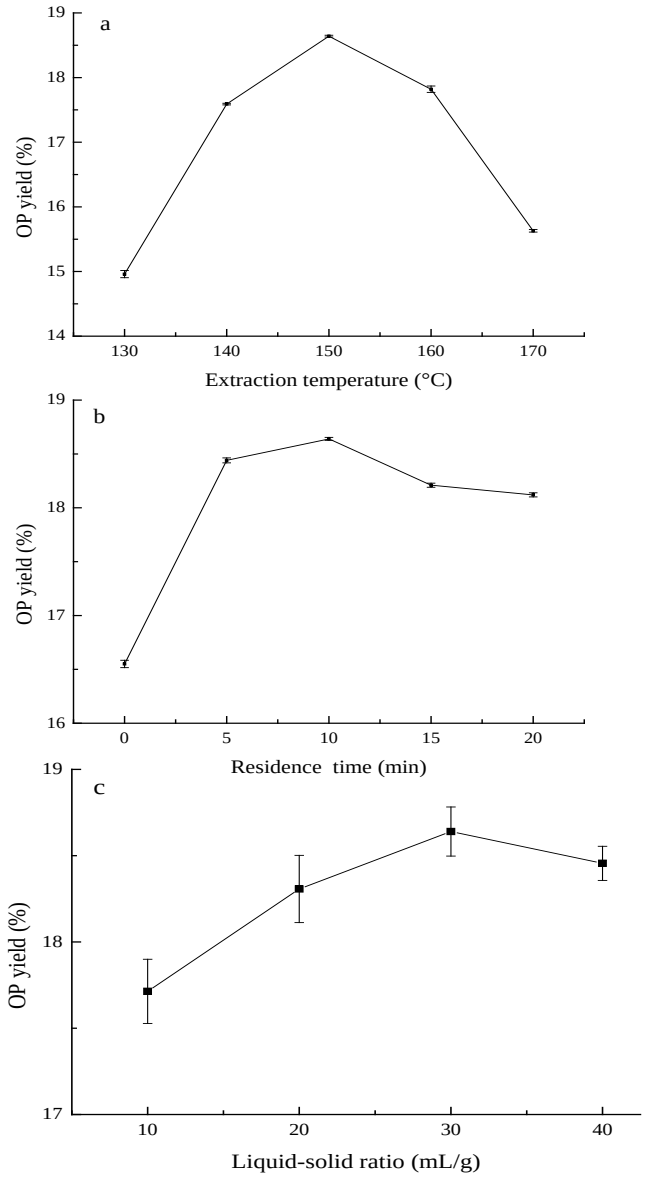


Fig. 2-2 Effect of different temperature (a), residence time (b) and liquid-solid ratio (c) on the yield of OP (n = 3). (a) 10 min of residence time and 30 mL/g of liquid-solid ratio; (b). 150°C of temperature and 30 mL/g of liquid-solid ratio; (c)10 min of residence time and 150°C of temperature.

2.3.1 Optimization of OP production from okara by using SWT

According to our preliminary results (Fig. 2-2), SWT conditions including temperature of 130-170°C, residence time of 5-15 min and liquid-solid ratio of 20-40 mL/g were chosen for optimization of SWT conditions by using RSM experiments in this study.

(1) Model fitting and statistical analysis

Coded factor levels and real values of the three process variables, including temperature (X_1), residence time (X_2) and liquid-solid ratio (X_3) were evaluated based on OP yield as listed in Table 2-2.

Table 2-2 Box-Behnken design matrix of the three variables (coded and uncoded units) and results of OP yield (*n=3*).

Run	X_1	X_2	X_3	OP yield (%)	
	(°C)	(min)	(mL/g)	Actual	Predicted
1	1 (170)	-1 (5)	0 (30)	15.59	15.73
2	0 (150)	0 (10)	0 (30)	18.60	18.64
3	-1 (130)	1 (15)	0 (30)	17.05	16.91
4	-1 (130)	-1 (5)	0 (30)	13.42	13.27
5	0 (150)	0 (10)	0 (30)	18.76	18.64
6	0 (150)	-1 (5)	-1 (20)	17.43	17.42
7	1 (170)	0 (10)	1 (40)	15.86	15.70
8	0 (150)	1 (15)	-1 (20)	17.97	17.95
9	1 (170)	1 (15)	0 (30)	12.61	12.76
10	1 (170)	0 (10)	-1 (20)	13.57	13.45
11	-1 (130)	0 (10)	-1 (20)	15.95	16.11
12	-1 (130)	0 (10)	1 (40)	14.62	14.74
13	0 (150)	0 (10)	0 (30)	18.45	18.64
14	0 (150)	-1 (5)	1 (40)	18.04	18.62
15	0 (150)	0 (10)	0 (30)	18.72	18.64
16	0 (150)	0 (10)	0 (30)	18.67	18.64
17	0 (150)	1 (15)	1 (40)	18.18	18.19

Table 2-2 also shows the results from the regression analysis using the Box-Behnken design matrix of the three variables (17 runs) in order to achieve an optimal region under the designed experimental conditions. By applying multiple regression

analysis (ANOVA) on the experimental data, the values of the regression coefficients were calculated, and the relationship between the response variable (Y, OP yield) and test variables (X_1, X_2 and X_3) were related by the following second-order polynomial equation (Eq. (2-6)):

$$Y = 18.64 - 0.43X_1 + 0.17X_2 + 0.22X_3 - 1.65X_1X_2 + 0.91X_1X_3 - 3.44X_1^2 - 0.53X_2^2 \quad (2\text{-}6)$$

Table 2-3 Analysis of variance (ANOVA) of the response surface quadratic model for the effects of temperature (X_1), residence time (X_2) and liquid-solid ratio (X_3) on OP yield.

Source	Sum of Squares	Df	Mean Square	*F*-value	*P*-value	Significant
Model	69.01	9	7.67	242.24	< 0.0001	****
X_1	1.45	1	1.45	45.92	0.0003	****
X_2	0.22	1	0.22	6.99	0.0333	**
X_3	0.40	1	0.40	12.51	0.0095	***
X_1X_2	10.92	1	10.92	345.08	< 0.0001	****
X_1X_3	3.28	1	3.28	103.50	< 0.0001	****
X_2X_3	0.04	1	0.04	1.26	0.2980	*
X_1^2	49.79	1	49.79	1572.95	< 0.0001	****
X_2^2	1.20	1	1.20	37.90	0.0005	****
X_3^2	0.17	1	0.17	5.39	0.0533	*
Residual	0.22	7	0.03			
Lack of Fit	0.16	3	0.05	3.64	0.1220	*
Pure Error	0.06	4	0.01			
Cor Total	69.23	16				
SD	1.78		R^2	0.9968		
Mean	166.76		*Adj.* R^2	0.9927		
C.V. (%)	1.07		*Pred.* R^2	0.9613		
Press	267.87		*Adequate precision*	43.0765		

**Significant, $P < 0.05$.

***Very significant, $P < 0.01$

****Highly significant, $P < 0.001$.

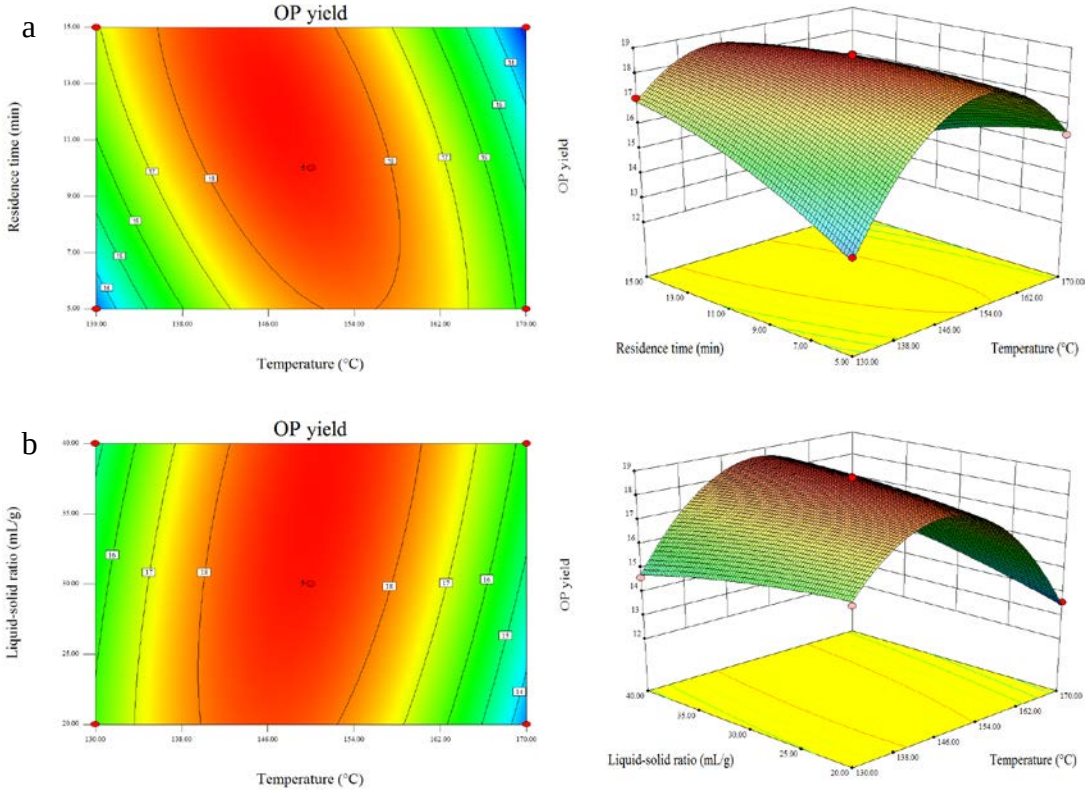
*Non-significant, $P > 0.05$.

Tables 2-3 lists the results of the statistical significance of the model equation (F-test), the significance of each coefficient calculated (*F*-value and *p*-value) and the analysis of variance for the response surface quadratic model. As seen, the *F*-value

(242.24) with a low probability value ($p < 0.0001$) implies a strong significance for the regression model. The goodness of fit of data to the model was also confirmed by the multiple correlation coefficient ($R^2 = 0.9968$), indicating that the sample variation of 99.68% for OP yield was statistically significant. However, as Samavati (2013) pointed out that high R^2 nearly equal to 1 is not sufficient to express the accuracy and general availability of the polynomial model. Hence, *Adj* R^2 (adjusted determination coefficient) was implemented to be an additional variable for R^2. The value of *Adj* R^2 (0.9927) implies that only less than 0.8% of the total variations are not included in this model, signaling the extraordinary accuracy of the developed model. Meanwhile, a distinguished value of predicted multiple correlation coefficient *Pred.* R^2 (0.9613) also indicates a strong correlation between the actual and predicted results. The lack-of-fit measures the failure of the model to represent the data in the experimental domain at points which do not appear in the regression (Chen, Zhou & Zhang, 2014). The *F*-value of 3.64 and *p*-value of 0.1220 for OP yield show that it is not significantly relative to the pure error due to noise. Besides, the "Adequate precision", as the measurement of the signal to noise ratio, is 43.0765, meaning that the present model could be used to navigate the design space under any combination of values of the variables. Additionally, 1.07 of coefficient of variation (C.V.) was calculated for OP yield to express the standard deviation as a percentage of the mean values, which proves that this model possesses high precision and good reliability of the experimental values. The *P*-value is an index to examine the significance of each coefficient, which in turn indicates the interaction strength between each independent variable (Zheng et al., 2014). The levels of significance of diverse coefficients were marked with asterisks in Table 2-3. In short, the present model could be adequately well for navigating the design space.

It can be seen that one linear coefficient (X_1), two cross product coefficients (X_1X_2, X_1X_3) and two quadratic term coefficients (X_1^2, X_2^2) are highly significant with very small P values ($p < 0.001$). A linear coefficient (X_3) is very significant ($p < 0.01$). Another linear coefficient, X_2 is also significant ($p < 0.05$). The other term coefficients are not significant ($p > 0.05$). The full model as Eq. (2-6) can be used to analyze the three-dimensional and contour plots and predict the relationships between the independent and dependent variables.

(2) Analysis of response surfaces



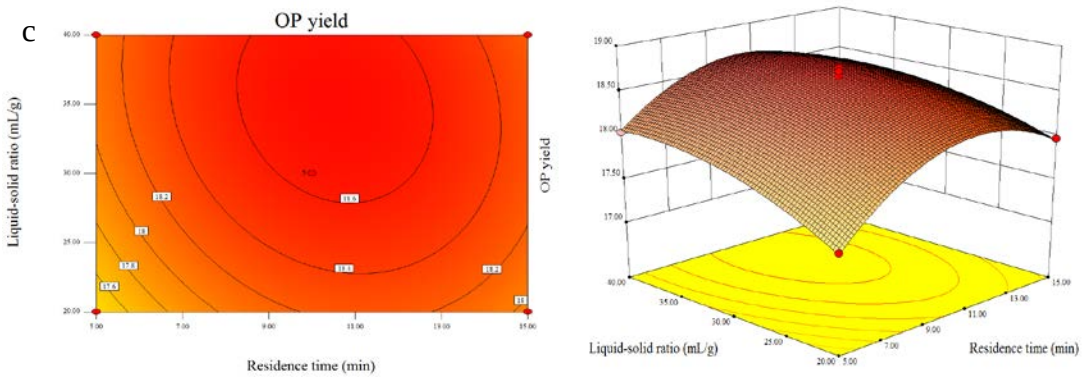


Fig. 2-3 Response surface (3-D) and contours plots for the effects of (a) temperature and residence time, (b) temperature and liquid-solid ratio and (c) residence time and liquid-solid ratio on extraction yield of OP.

Three-dimensional response surface plots and two-dimensional contour plots simulated by Design-Expert software (version 8.0.5) based on the regression model Eq. (2-6) were established to evaluate the effects of variables on the response. Fig. 2-3 visually illustrates the type of interactions between two test parameters on the OP yield. In order to identify the optimum values of the independent variables, two continuous variables are generated with the other one variable fixed at constant at the center value of the testing ranges (respectively zero level). The circular and elliptical contour plots indicate the significance of the mutual interactions. In contrast to the negligible effects shown by circular contour plots, elliptical contour plots imply the significant interactions between the independent variables.

The plots in Fig. 2-3a illustrate the variation of OP yield under varying temperature and residence time at a fixed liquid-solid ratio (30 mL/g). It clearly demonstrates that the OP yield firstly increased as the temperature increased from 130 to 148.3°C. Nevertheless, the yield was rapidly decreased after the peak, implying that an elevated temperature could induce the degradation and accelerate the hydrolysis of yielded polysaccharides. Besides, the tendency of effect of residence time was delineated as a parabola which peaked at 11.3 min, revealing that further extending of residence time

would not improve the yield of polysaccharides. In addition, the elliptical contour plot signals a strong interaction between these two variables.

The effects of temperature and liquid-solid ratio on the yield of OP are depicted in Fig. 2-3b (residence time was fixed at 10 min). Obviously, the maximum OP yield was achieved when the temperature and liquid-solid ratio were 148.3°C and 33.0 mL/g, respectively. In particular, for the liquid-solid ratio, a gradual increase in OP yield appeared from 20 to 33.0 mL/g. After the peak, the OP yield was observed to be insensitive to further increase in liquid-solid ratio, indicating that the accumulation of water was adequate for the binding of polysaccharides from okara. Furthermore, according to the elliptical contour plot, the interaction between the temperature and liquid-solid ratio is significant. Fig. 2-3c displays the interaction between the residence time and the liquid-solid ratio. It is worth noting that the rounded contour plot and the smooth surface imply a two-dimensional contour plot representing insignificant interactions between the tested variables ($p > 0.05$, Table 2-3).

Based on desirability function approach by the response surface analysis above, the optimum SWT conditions for OP yield were determined as follows: temperature of 148.3°C, residence time of 11.3 min and liquid-solid ratio of 33.0 mL/g. A maximum response, 18.71% of OP yield, with a desirability value of 0.992 can be predicted.

(3) Verification of the predictive model

To validate the adequacy of the model predictions and avoid large deviation, a verification experiment was carried out under the obtained optimal conditions (i.e. temperature of 148°C, residence time of 11.3 min, and liquid-solid ratio of 33.0 mL/g). The experimental yield of OP was 18.68 ± 0.24% (n = 3), which is in good agreement with the correspondingly predicted value (18.71%). The verification test definitely

reveals that the regression model is satisfactorily accurate for the production of OP by using SWT.

2.3.2 Characterization of yielded OP

(1) Monosaccharides found in OP produced from okara by SWT

As mentioned before, water can act as an excellent extraction solvent under SWT conditions, under which the extracted OP might be further decomposed to lower molecular monosaccharides. In this study, HPAEC-PAD was applied to quantify monosaccharides contained in the OP obtained under the above optimal SWT conditions (Fig. 2-4). Results show that a wide range of monosaccharides are detectable in the OP obtained in this study, including rhamnose, arabinose, galactose, glucose, xylose, mannose, galacturonic acid and glucuronic acid. Their retention time was determined to be 4.78, 5.42, 6.78, 7.34, 11.78, 12.43, 29.25 and 30.61 min, and their molar percentage in the OP was 4.39%, 30.28%, 56.67%, 1.41%, 3.40%, 0.34%, 3.26% and 0.24%, respectively. Clearly, the yielded OP contains some acidic saccharides which are beneficial for the health of human beings or animals (Singdevsachan et al., 2016). Among all the monosaccharides determined, galactose and arabinose are the main ones, amounting to 86.95% (molar percentage) in OP. These monosaccharides detected might contribute to the bioactivities of OP produced to some extent. The HPSEC-RID elution profile obviously showed one a single and symmetrically sharp peak (Fig. 2-5). According to the results achieved by HPSEC-MALLS-RI system, the M_w value of OP was 236.61 kDa. These molecular characteristics parameters played an important role in investigating the structure of polysaccharides.

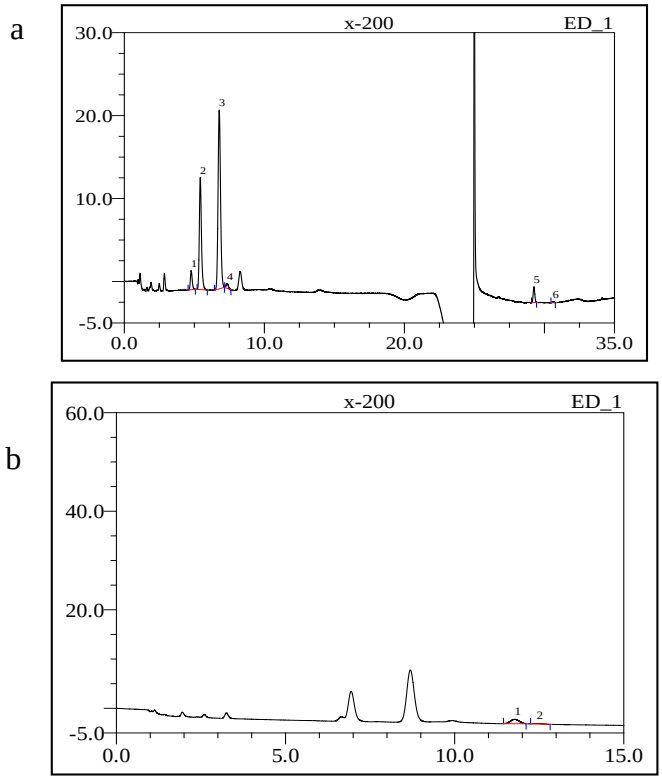


Fig. 2-4 HPAEC-PAD chromatogram profiles of the monosaccharides from okara polysaccharide sample. (a) Peak identity: 1, Rhamnose; 2, Arabinose; 3, Galactose; 4, Glucose; 5, Galacturonic acid; 6, Glucuronic acid. (b) Peak identity: 1, Xylose; 2, Mannose.

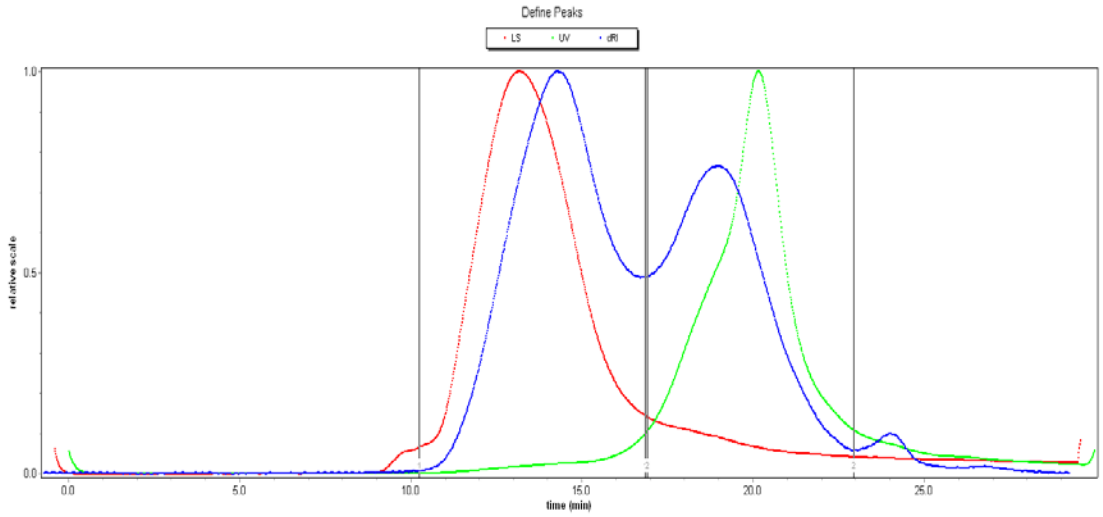


Fig. 2-5 GPC chromatogram of OP for molecular weight determination with HPSEC-MALLS-RID system. Peak 1 (10.261-16.865 min): calculated mass, 8.3685 × 10^{-5} g; mass fraction, 56.4230%; dn/dc, 0.135 ml/g; M_w, 3.69 ×10^5 (± 0.7%) Da, M_w/M_n, 1.42 (± 1.00%). Peak 2 (16.927 - 22.955 min): calculated mass, 6.4632 × 10^{-5} g; mass fraction, 43.5770%; dn/dc, 0.135 ml/g; M_w, 6.558 × 10^4 (± 3%) Da, M_w/M_n, 1.152 (± 4%). LS: laser light scattering; dRI: refractive light; UV: ultraviolet absorption detector.

(2) FT-IR analysis on yielded OP

The absorption bands within the range of 3600-3300 cm^{-1}, 3000-2800 cm^{-1}, and 1100-1000 cm^{-1} are the characteristic absorption peaks of polysaccharides (Yin et al., 2016). Results from the FT-IR spectrums of OP extracted by HWE and SWT (Fig. 2-6) fitted the typical pattern of polysaccharides well, which indicated that subcritical water technology did not destroy the polysaccharide structure. Specifically, SWT-OP was a typical polysaccharide with a strong and wide band around 3367 cm^{-1} for O–H stretching vibrations (Zha et al., 2014) and a relatively weak absorption peak at 2931 cm^{-1} for C–H stretching vibrations including CH-, CH_2- and CH_3-, respectively (Li & Wang, 2016). Absorption at 1733 cm^{-1} can be attributed to carbonyl groups bending vibration. Band at 1646 cm^{-1} may be derived from the bending vibration of O–H, which also signals the existence of hydroxyl groups in the yielded OP (Li & Shah, 2014). The signal at 1540 cm^{-1} corresponds to the presence of bending vibrations of N–H groups. These two bands indicate the residence of protein in OP (Radzki et al., 2016). The absorptions around 1418 cm^{-1} reflect CH_2 and OH bonding (Sun et al., 2014). The peak around 1244 cm^{-1} is regarded to be unsymmetrical carbonyl stretching (Zhao et al., 2013). The main absorptions of C–O stretching (1078 cm^{-1} and 1041 cm^{-1}) show that the characteristics of sugar structures are pyranose configuration (Shao et al., 2014), and the characteristic band at 891 cm^{-1} indicates agar specific characteristics, which are mainly associated with C–H bending at the anomeric carbon of 3-linked-β-d-galactose residue (Wongprasert et al., 2014).

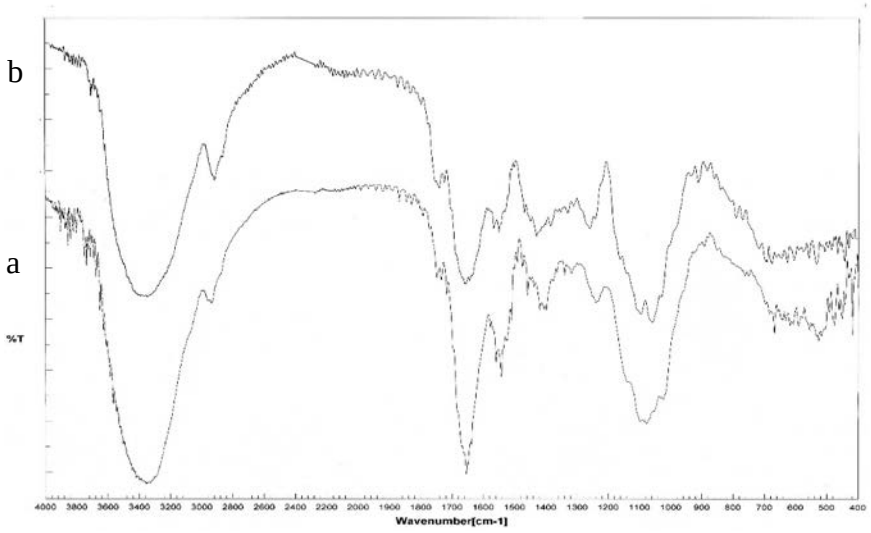
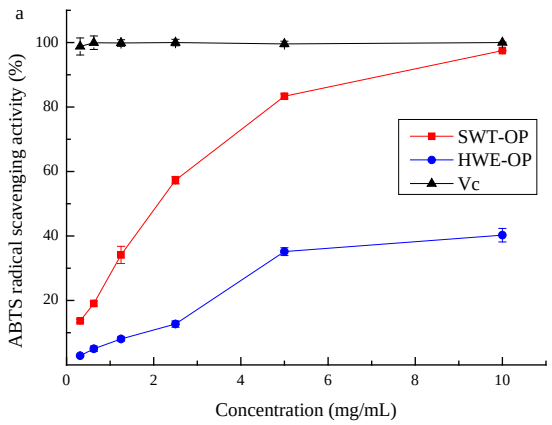


Fig. 2-6 FT-IR spectrum of the OP obtained from okara by using HWE (a) and SWT (b) under optimal operation conditions.

2.3.3 Antioxidant activity assay

The antioxidant activities of compounds are attributable to various reactions and mechanisms, such as radical scavenging, reductive capacity, prevention of chain initiation, and binding of transition metal ion catalysts (Frankel & Meyer, 2000). In the present study, the antioxidant activities of OP were evaluated by scavenging ABTS and hydroxyl radicals.



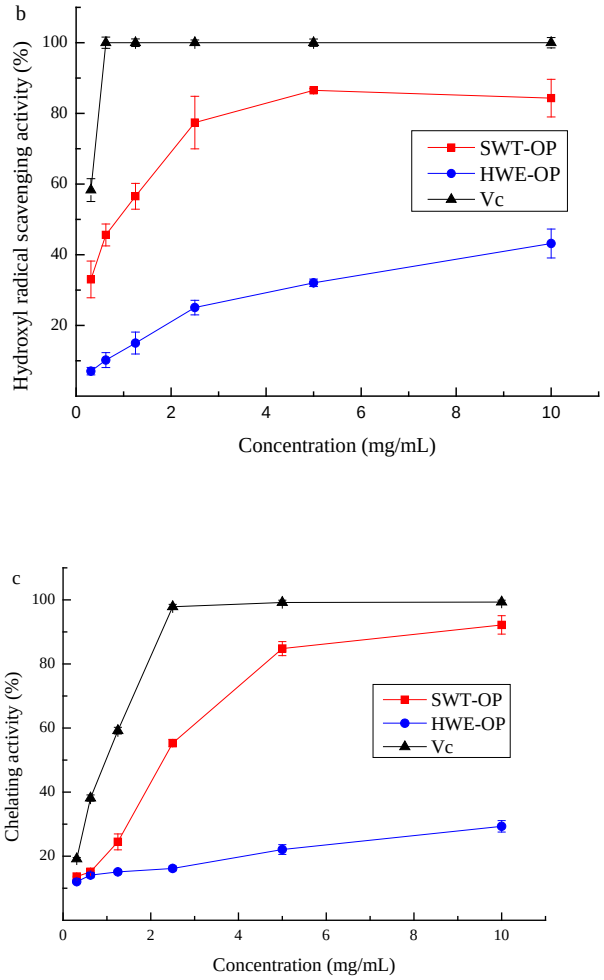


Fig. 2-7 Antioxidant activities of OP from okara by using subcritical water technology (SWT) and hot water extraction (HWE), which are labeled as SWT-OP and HWE-OP, respectively. (a) Scavenging activity on ABTS radicals; (b) Scavenging activity on hydroxyl radicals; (c) chelating activity. Values are expressed as means ± S.D. (n = 3).

Antioxidants can eliminate the radicals produced by activation of metmyoglobin with hydrogen peroxide in the presence of ABTS (Re et al., 1999), which provides the fundamental basis of spectrophotometric methods that have been applied to the measurement of the total antioxidant power of single ingredient and complex compounds from various plants (Gülçin et al., 2010). As shown in Fig. 2-7a, compared to HWE-OP, SWT-OP showed much stronger ABTS radical scavenging activity and its activity increased from 13.63% to 97.52% in a dose-dependent manner when

concentration increased from 0.3125 to 10.00 mg/mL. IC_{50} value (mg/mL), the concentration at which 50% inhibition *in vitro* occurs, can be used to indicate the bioactivity of the tested compounds, which are obtained by interpolation from the linear regression analysis. In this study, the IC_{50} value of SWT-OP was 1.70 ± 0.055 mg/mL (Table 2-4).

Hydroxyl radicals, one of the most commonly encountered and dangerous free radicals in cells, can react with the overwhelming proportion of biological macromolecules by a series of free radical chain reactions and further induce tissue damage and cell death (Rollet-Labelle et al., 1998; Sakanaka et al., 2005). Generally, generation inhibition and scavenging power are two primary aspects of anti-oxidation to control reactive oxygen species for the protection of living systems. The hydroxyl radicals scavenging abilities of SWT-OP and HWE-OP are illustrated in Fig. 2-7b. Although the scavenging activity of OP was lower than Vc at all tested concentrations, SWT-OP exhibited stronger inhibition ability on hydroxyl radicals than HWE-OP, which increased significantly from 33.02% to 86.54% when OP concentration was increased from 0.3125 to 5 mg/mL, and kept stable at higher concentrations. Its IC_{50} value was determined as 0.75 ± 0.11 mg/mL. Interestingly, SWT-OP possessed a relatively higher scavenging capability on hydroxyl radicals at lower concentrations, demonstrating that SWT-OP has an enhanced sensitivity to radicals scavenging. Results from this study indicate that the polysaccharides obtained from okara under subcritical water conditions could be further developed as a powerful hydroxyl radical scavenger.

2.3.4 Comparison between SWT and HWE

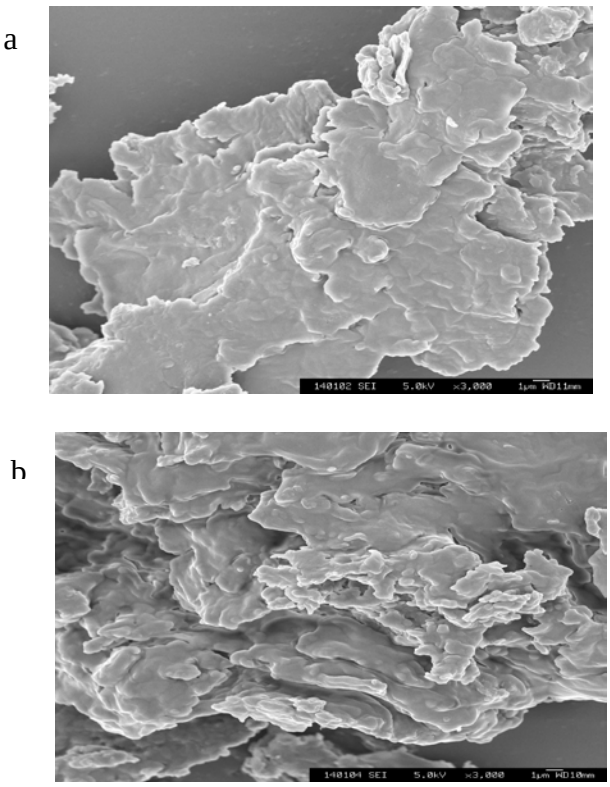


Fig. 2-8 SEM images of okara surfaces after using HWE (a) and SWT (b). HWE: temperature of 100°C, residence time of 120 min and liquid-solid ratio of 30:1; SWT: temperature of 148°C, residence time of 11.3 min and liquid-solid ratio of 33:1.

Being an ideal visual technique to characterize and visualize the elemental composition of a specimen, scanning electron microscopy (SEM) was conducted to analyze the surface and structural features of okara treated by HWE and SWT conditions (Fig. 2-8). As shown in Fig. 2-8a, the rough surface of okara after HWE had rigid and highly ordered multilayer structure of solid fibrils which might be responsible for the prevention of moisture loss, the protection of cell structures and the limitation of cellulose conversion. Moreover, smooth edges were also displayed, which was probably correlated with stabilization. However, after SWT treatment, a range of discrete and decrescent macro fibrils appeared on the cell surfaces meanwhile more

internal areas and porous layers were exposed (Fig. 2-8b). Besides, rough edges were shaped over the okara strand surfaces resembling a sharp blade, indicating some elements on the structure were partially removed.

Table 2-4 summaries the OP yields from okara by using HWE and SWT and their antioxidant activities. The results showed that SWT can achieve a much higher OP yield of 18.68% within a shorter reaction time, in comparison to 2.44% by HWE (nearly 7.7 times). Besides, HWE-OP exhibited weaker scavenging activities on ABTS (IC_{50} = 13.06 mg/mL) and hydroxyl radicals (IC_{50} = 14.71 mg/mL) than SWT-OP, which are in accordance with unfermented soybean curd residue (Li et al., 2013). It is likely that the structure of cells wall can be dramatically destroyed under high temperature and pressure conditions, which accelerates mass transfer from the solid phase into the water. Additionally, during this hydrothermal process some shorter chain carbohydrates generated from OP, partially manifested by the detected monosaccharides in this work, are capable of targeting free radicals and increasing the interactions, resulting in its enhanced antioxidant activities. Therefore, SWT, as an environmentally friendly processing technology, cannot only efficiently enhance the production of OP, but also probably preserve and enhance the bioactivity of OP.

Table 2-4 Comparison of yields and antioxidant activities of OP obtained by HWE and SWT.

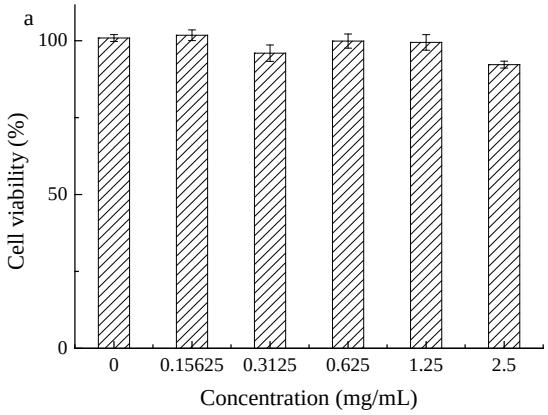
Method	Operation conditions			OP Yield (%)	IC_{50}	
	Temperature (°C)	Residence time (min)	Liquid-solid ratio (g/mL)		ABTS mg/mL	Hydroxyl mg/mL
HWE	100	120	30:1	2.44	13.06	14.71
SWT	148	11.3	33:1	18.76	1.70	0.75

The data are the mean of triplicate measurements.

HWE- hot water extraction, SWT- subcritical water technology

2.3.5 Cell assays

The non-toxicity of OP on TIG-3 cells and the anti-proliferative activity of OP against the growth U2OS cells were investigated using the MTT assay. As shown in Fig. 2.9a, cells viability was not dramatically decreased after drugs added and essentially unchanged. Slightly cells death should be considered as a result of the normal physiological metabolism of fibroblast cells themselves. The cell viability of TIG-3 cells was 92.23 ± 1.12% at the concentration of 2.5 mg/mL, indicating that OP had no cytotoxicity and could be a potential bioactive ingredient. The inhibition effect on U2OS cells was summarized in Fig. 2.9b. The results showed that OP possessed a dose-dependent activity within the concentration of 0.15625-2.5 mg/mL. Even though it was reported that molecular weight, structure of the polymeric backbone, chemical composition and degree of branching could affect the anti-proliferation capacity of polysaccharides, the mechanism of the anti-proliferative activity exerted by polysaccharides from okara extracted by using SWT was still not fully understood and needed further study.



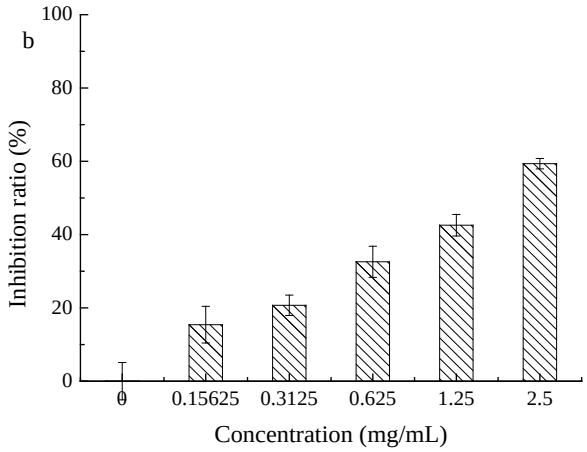


Fig. 2-9 Cell assays for OP by using SWT under the optimum conditions (temperature of 148.3°C, residence time of 11.3 min and liquid-solid ratio of 33:1). (a) The non-toxicity effect of OP on growth of TIG-3 human normal fibroblast cells for 72 hours. (b) The anti-proliferation of OP against the growth of U2OS human osteosarcoma cells for 72 hours.

2.4 Summary

In the present study, SWT was applied to produce OP from okara and the yielded OP was evaluated via *in vitro* antioxidant activity. OP yield was optimized using BBD under RSM and the maximum experimental OP yield was achieved with optimal conditions being determined. Besides, the FT-IR spectrum of OP clearly shows the characteristic functional groups of polysaccharides, and the obtained OP contains some monosaccharides like galactose and arabinose. The radical scavenging activities of OP on ABTS and hydroxyl were determined. In comparison to HWE, SWT can achieve higher OP yield and its OP possesses higher antioxidant activities. Consequently, subcritical water technology is regarded as a promising technology for polysaccharides extraction and production from okara, yielding much more OP with strong antioxidant activities.

Chapter 3 Thermal processing effects on the chemical constituents and antioxidant activity of extracts from okara

3.1 Introduction

As mentioned in 1-1, okara is rich in bioactive compounds including dietary fiber, protein, polysaccharides, phenolic compounds, many of which have been claimed to contribute to the antioxidant activity due to their redox properties derived from various possible mechanisms: free-radical-scavenging activity, transition-metal-chelating activity, and/or singlet-oxygen-quenching capacity (Yao & Ren, 2011). For instance, phenolic compounds play an important role in stabilizing lipid peroxidation and inhibiting various types of oxidizing enzymes. Nevertheless, the differences in the structures and substitutions may influence the phenoxyl radical stability and impair the antioxidant properties.

It is generally known that heating extraction is an essential processing procedure, which is attributed to the oxidation, thermal degradation, and leaching of bioactive compounds from fresh vegetables. Depending on the morphology and nutritional properties of raw materials, different heating conditions (e.g., heating duration and temperatures) have either positive or negative effects on the antioxidant properties of vegetables (Sharma et al., 2015).

However, no relevant reports on the effects of thermal effects on bioactive compounds, antioxidant activity in okara under subcritical water conditions. Therefore, the objective of this chapter was to investigate the effects of different temperatures under subcritical water extraction conditions on a range of potentially health-related chemical constituents and antioxidant capacities, in order to evaluate the potential of

SWT for the production of high bioactive extracts from okara.

3.2 Materials and methods

3.2.1 Chemicals and standard solutions

Ascorbic acid, Folin–Ciocalteu, ethanol, sodium carbonate, gallic acid, sodium nitrite, aluminium nitrate, sodium hydroxide, disodium hydrogen phosphate, potassium persulphate, sodium dihydrogen phosphate, phenol and D-glucose were purchased from Wako Pure Chemical (Osaka, Japan). 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), 2,2′--azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were purchased from Sigma-Aldrich, Inc. (Saint Louis, MO, USA). All the other chemical reagents were of analytical grade.

3.2.2 Sample preparation and extraction procedure

The pretreatment method of okara was as same as that previously mentioned (2.2.2). Pretreated okara powder was placed in the apparatus (MMS-200, OMLABO, JAPAN) and extracted in triplicate at the temperature range of 160 to 230°C, with a fixed liquid-solid ratio of 30 mL/g for a residence time of 10 min. All the extracts were filtered and evaporated under vacuum to dryness. The concentrated samples were lyophilized. All of them were kept at 4°C in the dark before use.

3.2.3 Determination of total organic carbon (TOC)

TOC was measured by a TOC analyzer (TOC-VCSN, Shimadzu, Japan) after the samples being centrifuged and filtrated through 0.45 μm membrane.

3.2.4 Determination of total sugar

The total sugar was determined by the phenol-sulfuric acid method with some modifications (Mecozzi, 2005). The color reaction was initiated by mixing 1 mL of solution with 0.5 mL of a 5% phenol solution and 2.5 mL of concentrated sulfuric acid, and the reaction mixture was incubated in a boiling water bath for 15 min. After cooling to room temperature, the optical density (O.D.) of the mixture was determined at 490 nm and the total carbohydrate content were calculated with D-glucose as a standard. The results were expressed as milligram of D-glucose equivalent per gram of okara (Shi et al., 2012).

3.2.5 Determination of polysaccharides

The method of determining polysaccharides was the same as that mentioned in 2.2.3.

3.2.6 Analysis of total phenolic content

Total phenolic content (TPC) was determined on the base of the Folin–Ciocalteu colorimetric method (Miguel et al., 2010). The sample (0.5 mL) and 2 mL of sodium carbonate (75 g/L) were added to 2.5 mL of 10% (w/v) Folin–Ciocalteu reagent. After 30 min of reaction at room temperature (intermittent shaking for color development), the absorbance was measured at 765 nm. The TPC was determined from the linear equation of a standard curve prepared with gallic acid. The content of total phenolic content was calculated as mean ± SD (n=3) and expressed as mg gallic acid equivalents (GAE)/g dry weight.

3.2.7 Analysis of total flavonoid content

The total flavonoid content (TFC) in extract were measured by a colorimetric assay (Zhishen et al., 1999). The extract (5 mL) was added to a 10 mL flask, and then 5% $NaNO_2$ solution (0.3 mL) was added. After mixed well, the solution was allowed to stand for 6 min at room temperature; and 5% $Al(NO_3)_3$ solution (0.3 mL) was added to the flask, mixed well and kept for 6 min at room temperature. At last 4% NaOH solution (4.4 mL) was added, mixed well and kept for 12 min at room temperature. Absorbance was read on a UV-spectrophotometer at 510 nm, and the total flavonoid contents (%) were estimated using calibration curves. Total flavonoid content in samples was calculated from a calibration curve ($R^2 = 0.999$) using rutin and expressed as mg of rutin equivalent (RE) per g of dry weight.

3.2.8 Antioxidant activity assays

(1) DPPH radical scavenging activity assay

DPPH radical scavenging activity of extractions was evaluated according to a literature procedure (Li et al., 2013) with slight modifications. Aliquots (0.5 mL) of various concentrations (0.3125-10.00 mg/mL) of solutions were mixed with 3 mL (25 μg/mL) of a MeOH solution of DPPH and then were shaken vigorously and allowed to stand in the dark for 30 min. The absorbance was measured with a spectrophotometer at 517 nm against a blank. Decrease of the DPPH solution absorbance indicated an increase of the DPPH radical scavenging activity. Ascorbic acid was invoked as positive controls. The DPPH radical scavenging activity was calculated according to the following equation:

$$\text{Scavenging activity (\%)} = (1 - A_1/A_0) \times 100\% \quad (3\text{-}1)$$

where A_0 is the absorbance without samples and A_1 the absorbance containing the

samples.

Results were expressed in millimole (mM) ascorbic acid equivalent (AAE) per 1 g of a sample on a dry weight basis for the further correlation analysis.

(2) ABTS radical scavenging activity assay

ABTS radical scavenging activities of extracts were measured using the methods mentioned in 2-2.5. Results were expressed in millimole (mM) ascorbic acid equivalent (AAE) per 1 g of a sample on a dry weight basis for the further correlation analysis.

3.3 Results and discussion

3.3.1 Extraction yields

(1) General

As other processing steps like milling, grinding, homogenization, extraction can recover and isolate phytochemicals from plant materials and is important for obtaining extracts with acceptable yields and strong antioxidant activity (Moure et al., 2001). Generally, extraction efficiency is associated with the chemical nature of phytochemicals, the extraction method used, sample particle size, the solvent used, as well as the presence of interfering substances (Stalikas, 2007). Besides, the yield of extraction depends on the solvent with varying polarity, pH, temperature, extraction time, and composition of the sample. According to previous studies, extraction temperature is one of the crucial parameters, especially under the subcritical condition.

Results of extraction of okara at various temperatures are presented in Table 3-1. As the most important parameter indication for decomposition of okara in the subcritical hydrolysis reaction, total organic carbon (TOC), and total sugar (TS) were measured. The results showed that the effect of subcritical temperature on TOC and TS at a reaction time of 10 min with a designed liquid-solid ratio of 30 mL/g. It could be

observed that the TOC was 29.83 ± 0.75% (160°C) and kept relatively stable until the extract at 180°C which produced the highest TOC 30.00 ± 0.73%. Then this sharply decreased to 20.64 ± 0.73% and remained around 20%, possibly owing to a weak hydrolysis reaction, pyrolysis, and gasification of the organic compounds (Pourali et al., 2009). The trend of TS content was similar to that of TOC. The highest content (210.36 ± 4.35 mg GE/g) was achieved from okara at 160°C, which was 5.31-fold of that obtained with boiling water for 2h (2-3.4). After that, the content of total sugar decreased somewhat by increasing temperature due to the final degradation of polysaccharides, which were also shown in the same Table 3-1. Total sugars of the aqueous phase comprise mixtures of poly-, oligo-, di-, and mono-saccharides. Under certain temperature, polysaccharides yield (145.61 ± 3.09 mg GA/g) was significantly higher than that extracted using HWE for a longer time, suggesting that subcritical treatment hydrolyzed them to significant amounts of water-soluble sugars (Pourali et al., 2009). Furthermore, correlation analyses were performed on TOC and TS, TS and polysaccharides of okara extracts, respectively. According to Pearson correlation analysis of TOC and TS, the correlation coefficient value was 0.899, which indicated that the relationship between TOC and TS was statistically significant. As for correlation analysis of TS and polysaccharides, a value (0.952) nearly closed to 1 was calculated, indicating that polysaccharides are the primary components of total sugar.

(2) Total phenolic content

Since soybeans contain various bioactive phytochemicals, we compared the total phenolic contents of water extracts under different extraction temperatures as compared to those of commercially available samples. Under alkaline conditions, Folin–Ciocalteu’s phenol reagent (yellow color) reacts with phenolic compounds and, consequently, a phenolate anion is formed by dissociation of a phenolic hydrogen atom

(Blois, 1958). This sequence of reversible one- or two-electron reduction reactions leads to blue-coloured chromophores being formed between phenolate and the FC reagent (Prior et al., 2005).

The total phenolic content (TPC) of various extracts in this study was determined based on the aforementioned reagent method. TPC values were calculated from the gallic acid standard calibration curve y = 0.0056x - 0.0003 with R^2 = 0.9995, where x is the absorbance and y is the concentration of gallic acid equivalents expressed as mg GAE/g. Significant differences were found for TPC among the extracts at various subcritical temperature. The TPC of various aqueous extracts were in the range of 23.32 ± 0.14 mg GAE/g to 76.18 ± 0.45 mg GAE/g under subcritical conditions (Table 3-1) when extracting temperature increased from 160°C to 220°C with other two conditions fixed (residence time of 10 min and liquid-solid ratio of 30 mL/g). Briefly, aqueous samples at 170, 180, 190, 200 and 210°C yielded TPC of 27.12 ± 0.37, 39.05 ± 0.46, 50.50 ± 0.54, 67.25 ± 0.73 and 71.14 ± 0.54 mg GAE/g, respectively. It was crucial to note that, on heating at 220°C for 30 minutes at a liquid-solid ratio of 30 mL/g, the highest level of TPC was markedly improved approximately 22 folds than that of the extract at ambient temperature for a longer residence time, 2 h. When the heating temperature rose to 230°C, a slight decrease (70.82 ± 0.65 mg GAE/g) occurred, mainly because of the accelerated interaction decomposition of target products under the elevated temperature. Hence, it can be concluded that SWT at 220°C was the most efficient condition among those investigated in this work for the extraction of phenolic compounds from okara. And all the results are in good agreement with the published results related with the total phenolic content of canola meal achieved by subcritical water technology (Hassas-Roudsari et al., 2009).

It is well-known that phenolic compounds have inhibitory effects on mutagenesis

and carcinogenesis in humans when ingested up to 1 g daily from a diet rich in fruits and vegetables (Mulero et al., 2015). The interests of phenolics are increasing in the food industry because they retard oxidative degradation of lipids and thereby improve the quality and nutritional value of food (Wojdyło et al., 2007). As effective natural antioxidants, phenolic compounds can be liberated from various plants by means of different processing procedures including sautéing, frying, boiling and roasting. The cleaving of the esterified and glycosylated bond or the formation of Maillard reaction during the heating process may be responsible for the increase in total phenolics after heating (Priecina & Karlina, 2013; Sharma et al., 2015). Numerous publications reported excellent linear correlations between “total phenolic content” and ‘‘antioxidant capacity” (Huang et al., 2005). The phenolic compounds may contribute directly to antioxidative action (Duh et al., 1999). Polyphenols are crucial plant constituents as a result of their scavenging ability because of their hydroxyl groups (Bendary et al., 2013) and markedly slow down the rate of conjugated diene formation at certain concentrations (Shukla et al., 2009).

(3) Total flavonoid content

Flavonoids are the naturally occurring polyphenols representing one of the most prevalent classes of compounds in vegetables, nuts, fruits, and beverages such as coffee, tea, and red wine (Pandey & Rizvi, 2009). The total flavonoid content (TFC) of different extracts in this study were analyzed according to Xie et al. (2015) with some modifications. TFC values were calculated from rutin standard calibration curve $y=0.0012x + 0.0027$ with $R^2 = 0.992$, where x is the absorbance and y is the concentration of rutin equivalents expressed as mg RE/g. The TFC of okara extracted at different temperatures are shown in Table 3-1. The present study showed the total flavonoid contents varied from 15.60 ± 0.87 (160°C) to 66.12 ± 0.69 mg RE/g (220°C)

with the difference of 4.24-fold in the initial flavonoid contents. By contrast, aqueous extract at 220°C had the highest total flavonoid contents in comparison with that extracted at ambient temperature, which proved that thermal treatment played a positive role in the extraction of TFC. Furthermore, an increasing trend of TFC in other seven extracts was found as the temperature elevated, except for that at 230°C. Specifically, aqueous samples at 170, 180, 190, 200, 210 and 230°C yielded TPC of 18.27 ± 0.19, 20.31 ± 0.40, 28.12 ± 0.88, 49.00 ± 1.40, 58.12 ± 0.64 and 65.91 ± 0.66 mg RE/g, respectively. After extracting at a definite temperature (220°C), there was a moderate decrease in the TFC. The reason for the decrease in the total flavonoid at higher temperature could be because of the degradation of flavonoids, which possibly depends on the structure of particular flavonoids (Sharma et al., 2015). Our results are consistent with the findings of Ioku et al. (2001) who found that total flavonoid content increased after heating at a certain temperature and magnitude of time, whereas too much exposure to severe conditions reduced the content of total flavonoid content. Moreover, in most fruits and vegetables, flavonoids contain Ceglycoside bonds and exist as dimers and oligomers, and the industrial processing such as heating or boiling results in the formation of monomers by the hydrolysis of Ceglycosides bonds (Manach et al., 2004).

It was observed that the effect of extracting temperature on TFC is similar to that on TPC. A correlation analysis was performed on the phenolic content (TPC and TFC) of okara extracts. The Pearson Correlation between TPC and TFC assay was found to be 0.964, which indicates that flavonoids are the dominating phenolic group in okara. This result is similar to the extraction of phenolics from *Limnophila aromatic* (Do et al., 2014).

Table 3-1 Total organic carbon, total sugar, polysaccharides, total phenolic compounds and antioxidant activities of different okara extracts by using SWT.

Temperature (°C)	TOC (%)	Total Sugar (mg GE/g)	Polysaccharides (mg GAE/g)	TPC (mg GAE/g)	TFC (mg RE/g)	DPPH (mM AAE/g)	ABTS (mM AAE/g)
160	29.83 ± 0.75	210.36 ± 4.35	145.61 ± 3.09	23.32 ± 0.14	15.60 ± 0.87	0.85 ± 0.14	31.54 ± 0.73
170	28.27 ± 0.79	174.02 ± 2.41	92.41 ± 0.23	27.12 ± 0.37	18.27 ± 0.19	3.39 ± 0.27	45.00 ± 2.30
180	30.00 ± 0.73	163.77 ± 1.42	61.86 ± 2.98	39.05 ± 0.46	20.31 ± 0.40	7.31 ± 0.35	73.77 ± 1.15
190	20.64 ± 0.73	112.56 ± 6.14	48.86 ± 1.40	50.50 ± 0.54	28.12 ± 0.88	18.73 ± 1.15	103.28 ± 2.41
200	21.73 ± 0.35	89.16 ± 1.81	19.01 ± 1.14	67.25 ± 0.73	49.00 ± 1.40	23.75 ± 0.36	126.37 ± 0.88
210	22.17 ± 0.25	61.08 ± 2.09	3.13 ± 0.045	71.14 ± 0.54	58.12 ± 0.64	38.67 ± 1.14	156.19 ± 3.47
220	20.86 ± 0.20	39.09 ±0.93	0.54 ± 0.0091	76.18 ± 0.45	66.12 ± 0.69	43.43 ± 0.58	169.61 ± 9.70
230	20.30 ± 0.23	22.83 ± 0.44	0.30 ± 0.014	70.82 ± 0.65	65.91 ± 0.66	41.83 ± 1.53	177.12 ± 8.77

[a] Data are expressed as means ± standard deviation of triplicate samples.

[b] All extracts were obtained with two fixed variables (liquid-solid ratio of 30 mL/g and residence time of 10 min)

49

3.3.2 Antioxidant capacities

(1) Antioxidant capacity of okara extracts using SWT on DPPH radicals

Hydrogen atom transfer, single electron transfer and metal chelation are the three primary proposed mechanisms, through which the antioxidants can play pivotal roles in protecting cells against damage caused by free radical-induced oxidative stress (Leopoldini et al., 2011; Ozsoy et al., 2008). Since different trends have been found in various antioxidant activity assays, it is of necessity to evaluate and compare the antioxidant capacity of extracts by means of multiple assays rather than one single test. In this study, DPPH radical scavenging activity and ABTS radical scavenging activity were employed. DPPH radical is a stable free radical with a characteristic absorption maximum at 517 nm (Soare et al., 1997). Lipid autoxidation contributes to the initiation of chains of lipophilic radicals, of which DPPH radical is considered to be a typical model (Vani et al., 1997). DPPH accepts an electron or hydrogen radical species in the presence of hydrogen donors such as phenolic components, resulting in the formation of table diamagnetic molecules, DPPH-H, thereby inducing a visually noticeable discoloration of assay solutions absorption from purple to yellow (Lee et al., 2001). During this reaction, the reducing number of DPPH radical molecules was equal to the number of hydroxyl groups of the antioxidant (Bondent et al., 1997). Additionally, DPPH radical scavenging activity assay is sensitive enough to accommodate samples in a short period of time and detect active ingredients at low concentrations (Hseu et al., 2008). Therefore, the radical scavenging activity of hydrogen-donating antioxidants can be determined as a decrease in the absorbance of DPPH solution (Petlevski et al., 2013).

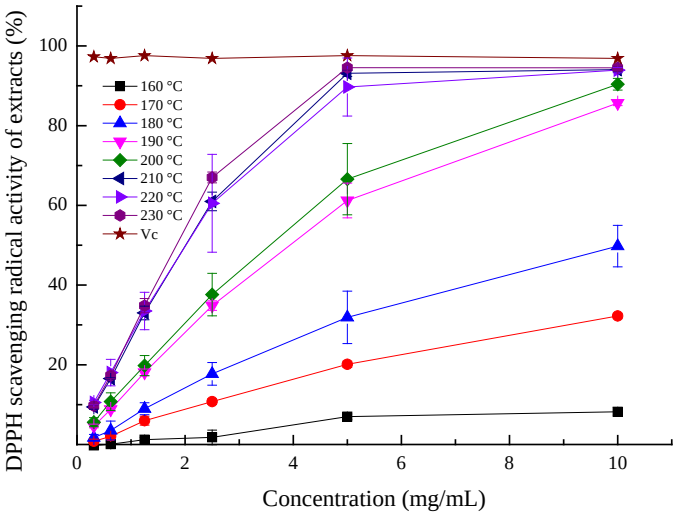


Fig. 3-1 DPPH scavenging radical effects of eight different aqueous extracts from okara by SWT and standard antioxidant (ascorbic acid). Data are expressed as means ± standard deviation of triplicate samples.

The DPPH scavenging radical effects of the eight different aqueous extracts from okara and standard antioxidant (ascorbic acid) were compared and shown in Fig. 3-1. In this study, ascorbic acid showed DPPH radical-scavenging activity of 97.30 ± 0.12% at the initial concentration of 0.3125 mg/mL, exhibiting excellent scavenging ability on DPPH radicals. And altered concentrations of all the SWT-extracts (0.3125, 0.625, 1.25, 2.5, 5 and 10 mg/mL) showed DPPH scavenging activities in a dose dependent manner. Specifically, When the concentration of aqueous extracts were 10 mg/mL, the scavenging rate was the highest, which were 8.22 ± 1.07% for the sample processed at 160°C, 32.24 ± 0.65% for the sample processed at 170°C, 49.77 ± 5.21% for the sample processed at 180°C, 85.65 ± 0.62% for the sample processed at 190°C, 90.38 ± 1.47% for the sample processed at 200°C, 94.08 ± 0.70% for the sample processed at 210°C, 93.94 ± 0.68% for the sample processed at 220°C, 94.51% for the sample processed at 230°C, respectively. In other words, the scavenging activity increased as the temperature elevated. Thermal treatment affected the DPPH scavenging activities of

aqueous extracts ($p < 0.05$). The results indicated that among these processing conditions, the thermal processing condition for samples processed at 230°C significantly improved the antioxidant activities of aqueous extract on DPPH radicals ($p < 0.05$), which is even equal to that of ascorbic acid in the lower concentration range (5-10 mg/mL). Furthermore, all aqueous extracts on DPPH assay followed an ascending the order of 160°C < 170°C < 180°C < 190°C < 200°C < 210°C < 230°C < 220°C (Table 3-1). It seems that extraction by SWT increases the quantity of antioxidant metabolites in the obtained extract and advances radical scavenging activity, rather than affecting their antioxidant activity. Another explanation for this phenomenon could be attributed to the hydrolysis of glycoside bonds of phenolic compounds thus increasing the number of phenolic hydroxyl groups and consequently the antioxidant activity of the extracts, taking account into TPC and TFC content in the extracts.

(2) Antioxidant capacity of okara extracts using SWT on ABTS radicals

ABTS forms a stable radical cation on reaction with potassium persulphate after keeping for 12 - 16 h in dark (Kumar & Rawat, 2013). Since the characteristic absorption spectrum of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) ABTS cation radical can be determined at 414, 645, 734 and 815 nm (Arnao et al., 1990; Miller et al., 1993) because of the presence of antioxidants (Villaño et al., 2004), common organic radical cation ($ABTS^{\cdot +}$) assay is one of the most widely methods applied to assess antioxidant activity (Rawat et al., 2011). Various systems including enzymatically using myoglobin (Rice-Evans & Miller, 1994) or horseradish peroxidase (Arnao et al., 1996), chemically with MnO_2 (Benavente-Garcıa et al., 2000), potassium persulfate (Re et al., 1999) or peroxide radicals and even electrochemically (van den Berg et al., 1999) can facilitate the generation of radicals from ABTS. The qualification

of being soluble in both organic and aqueous media and the stability in a wide pH range has contributed to the application of ABTS, estimating hydrophilic and lipophilic nature of the compound in sample (Kumar et al., 2012). In addition, other reports indicated that ABTS radical scavenging activity is more of accuracy, sensitivity and robustness for screening single ingredient and other complex antioxidant mixtures such as plant extracts, beverages, and biological fluids (Govindan & Muthukrishnan, 2013).

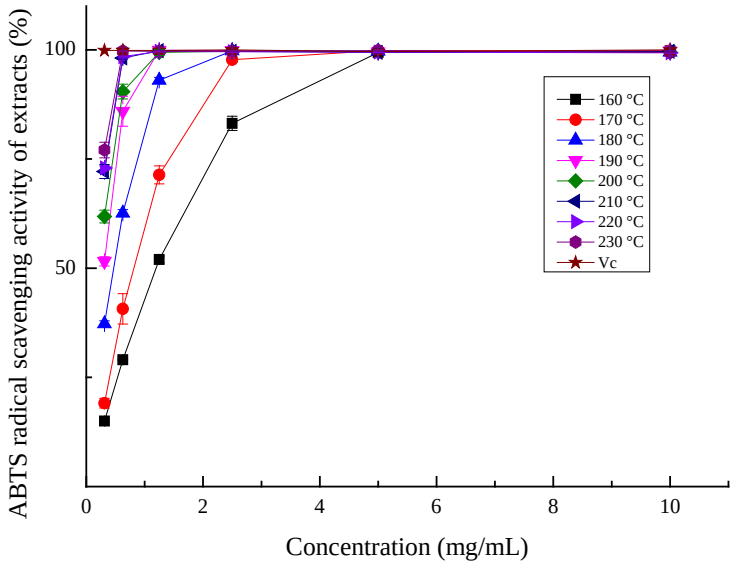


Fig. 3-2 ABTS scavenging radical effects of eight different aqueous extracts from okara by SWT and standard antioxidant (ascorbic acid). Data are expressed as means ± standard deviation of triplicate samples.

Eight extracts at various temperature levels were measured and compared for their free radical scavenging activities against ABTS radical cation and the results of the scavenging ability of SWT-extracts on ABTS free radicals were shown in Fig. 3-2. Apparently, all the extracts reduced the absorbance at 734 nm, and the concentration of the extracts was directly proportional to the reduction. In the ABTS radical scavenging assay, noteworthy differences among values of all the extracts were found ($p < 0.05$). As the control group, Ascorbic acid at a concentration of (0.3125- 10 mg/ml) was also

available to produce excellent inhibition of ABTS radicals. To be more specific, levels of ABTS radical scavenging activities followed a descending order of 230°C > 220°C > 210°C > 200°C > 190°C > 180°C > 170°C and 160°C with values of ranged from 77.06%, 72.94%, 72.13%, 61.83%, 51.57%, 37.27%, 19.10% and 15.01% at the initial concentration of 0.3125 mg/mL, respectively. When the concentration raised to 2.5 mg/mL, almost all the samples were equal to the effect produced by ascorbic acid at the same concentration, except for the sample processed at 160°C. Another result should be pointed out that the effect of scavenging capacity on ABTS radicals was as significant as that of ascorbic acid when the concentration of test solution was greater than 5 mg/mL, which indicated that there was no difference on ABTS radicals scavenging activity between aqueous extracts and Vc at a relatively higher concentration.

Also, it should be mentioned that DPPH and ABTS assays were conducted in ethanol and water media, respectively (Zhu et al., 2011). From a mechanistic perspective, the DPPH radical scavenging assay emphasizes the capacity of the extract transferring electrons or hydrogen atoms, while the ABTS radical scavenging activity could reflect the hydrogen donating and the chain-breaking capacity of the extract (Pérez-Jiménez et al., 2008). The order of ABTS radical scavenging activity was consistent with that of the DPPH radical, suggesting thermal treatment had positive effects on scavenging radicals dissolved in organic or aqueous solvent, to some extent.

3.3.3 Correlations among constituents and antioxidant activities.

In order to evaluate the suitability and reliability of the antioxidant assay for measurement of total antioxidant activity of extracts from okara by SWT, Pearson correlation coefficients between the antioxidant capacities (DPPH and ABTS) total

organic carbon, total sugar, polysaccharides, total phenolic content and total flavonoid content for all extracts prepared under different extraction temperatures were calculated and the results shown in Table 3-2. According to the results of the present study, significant correlations ($p < 0.05$) were detected in all cases, which revealed that values of antioxidant activity determined by two different methods were comparable and reliable.

TOC showed strongly linearrelationship with total sugar ($R = 0.899$; $R^2 = 0.808$, $p < 0.01$), and polysaccharides ($R = 0.832$; $R^2 = 0.692$, $p < 0.05$), which is in accordance with the fact that carbon element is essential in these two compounds. It should be noted that TOC, total sugar and polysaccharides was negatively correlated with two selected *in vitro* assays, suggesting there was no direct relation between the three aforementioned carbohydrates and antioxidant abilities under these subcritical water conditions. Too much exposure to elevated temperatures contributed to their degradation and inactivation. The correlation coefficient for DPPH and ABTS assay ($R = 0.988$; $R^2 = 0.976$, $p < 0.01$) indicated that the values of antioxidant activities assayed by these two different methods were significantly correlated, associating with spectrophotometry-based assay methods and elimination ability of the radical cation (Skotti et al., 2014).

Phenolic compounds in extracts, like other antioxidative compounds, are believed to account for a major portion of the antioxidant activity in many plants due to interaction with free radicals by acting as electro donor of hydrogen atmos. Measurements of the scavenging effects of DPPH and ABTS radicals showed that radical scavenging capacity increased with increase in TPC. Apparently, TPC exhibited strong correlations with both DPPH ($R = 0.962$; $R^2 = 0.925$, $p < 0.01$) and ABTS ($R = 0.980$; $R^2 = 0.960$, $p < 0.01$). Consistently, as main ingredients of TPC ($R = 0.959$; R^2

= 0.920, $p < 0.01$), TFC also has closed connections with both DPPH ($R = 0.982$; $R^2 = 0.945$, $p < 0.01$) and ABTS ($R = 0.973$; $R^2 = 0.947$, $p < 0.01$). The high correlations obtained in this work could suggest that the antioxidant activities of these extracts are resulted from phenolic compounds which contribute to the DPPH and ABTS scavenging activities. All these results are different from previous studies on *Amaranthus mantegazzianus* (Castel et al., 2014). Three factors may bring about the differences between the results of this study and other studies: (1) the difference in plant matrix; (2) the method and conditions of extraction (temperature, time and solvents) led to differences in compositions and antioxidant activities; (3) the difference of phenolic structures like possessing a higher number of hydroxyl groups (Do et al., 2014). It is worth noting that there must be some other soluble compounds existing in the extracts, like proteins, peptides, and pigments, might be also responsible for the antioxidant activity partly (Prior et al., 2005).

Consequently, the free radical scavenging ability of the extracts extracted by SWT might be significantly affected by the phenolic compounds, which is crucial to the ultimate antioxidant potency. Considering the high consumer demand owing to their purported beneficial health effects, okara byproducts can be utilized in the development of functional foods, as well as in health-promoting and pharmaceutical agents.

Table 3-2 Correlation coefficients between constitients and antioxidant activities of okara extracts by SWT.

R (R^2)	TOC	Total sugar	Polysaccharides	TPC	TFC	DPPH	ABTS
TOC	1						
Total sugar	0.899 (0.808)**	1					
Polysaccharides	0.815 (0.664)*	0.952 (0.906)**	1				
TPC	-0.878 (0.771)**	-0.967 (0.935)**	-0.959 (0.920)**	1			
TFC	-0.821 (0.674)*	-0.968 (0.937)**	-0.897 (0.805)**	0.959 (0.920)**	1		
DPPH	-0.857 (0.734)**	-0.981 (0.962)**	-0.914 (0.835)**	0.962 (0.925)**	0.982 (0.945)**	1	
ABTS	-0.872 (0.760)**	-0.993 (0.986)**	-0.954 (0.910)**	0.980 (0.960)**	0.973 (0.947)**	0.988 (0.976)**	1

R: correlation coefficient, R^2: coefficient of correlation.

* Significant at $p < 0.05$.

** Significant at $p < 0.01$

57

3.4 Summary

Thermal processing had significant effects on the compositions and antioxidant activities on okara extracts in this study. Specifically, TOC, total sugar and polysaccharides in extracts decreased under elevated temperature due to some reaction like pyrolysis and gasification. The highest yields of TPC and TFC were observed in the extract obtained as extraction temperature was 220°C with the other two fixed parameters (residence time of 10 min and liquid-solid ratio of 30 mL/g). Among the various aqueous extracts, all aqueous extracts showed high antioxidant activity, which indicated SWT played important roles in the increase of the antioxidant capacities of okara extracts on DPPH scavenging activities and ABTS assays. According to correlation comparisons, the total phenolic contents of the extracts were coherent with the antioxidant activities of the extracts, pending further analysis of their specific composition to explain the underlying mechanism. This finding could be useful to the disposal processing of okara by-products in the food industry.

Chapter 4 Optimization of subcritical water extraction of phenolic compounds from okara using response surface methodology

4.1 Introduction

According to Chapter 3, SWT had a positive effect on the extraction of phenolic compounds with high antioxidant activity within certain limits. Since high extraction efficiency influenced by extraction methods, solvent types, solvent concentration, extraction temperature and time is advantageous for an industrial process, it is worth figuring out the optimum conditions using SWT.

Response surface methodology (RSM) is an efficacy mathematical and statistical technique for analysis of empirical models that describes the effect of independent variables and their interactions on response variables, thereby optimizing the chemical process.

This chapter is to investigate the effects of SWT conditions (temperature, residence time and liquid-solid ratio), and to apply response surface methodology so as to optimize these conditions to obtain the highest total phenolic content (TPC), total flavonoid content (TFC) and the highest antioxidant activity (DPPH) of obtained aqueous extracts of dried okara

4.2 Materials and methods

4.2.1 Chemicals

Ascorbic acid (Vc), Folin–Ciocalteu, ethanol, sodium carbonate, gallic acid, sodium nitrite, aluminium nitrate, sodium hydroxide, disodium hydrogen phosphate and phenol were obtained from Wako Pure Chemical (Osaka, Japan). 2,2-Diphenyl-1-

picrylhydrazyl (DPPH) was purchased from Sigma–Aldrich , Inc. (Saint Louis, MO, USA). All chemicals were reagent grade and used without further purification. Aqueous solutions were prepared with distilled water.

4.2.2 Extraction procedure

The pretreatment method of okara was as same as that previously mentioned. 1 g pretreated okara powder was treated in batch mode in a MMS-200 reactor (OMLABO, JAPAN) in triplicate using subcritical water technology. All the extracts were filtered through Whatman No 1 and the filtrates were stored at 4^{o}C in the dark prior to analysis.

4.2.3 Analytical measurements

Total phenolic content and total flavonoid content were determined by the methods as mentioned in 3.2.6 and 3.2.7, respectively.

DPPH radical scavenging activity was evaluated as 3.2.8.1. The scavenging activity on DPPH radicals was expressed as IC_{50} value, which was defined as the concentration of the sample necessary to cause 50% inhibition, obtained by interpolation from a linear regression analysis.

4.2.4 Experimental design

In order to evaluate the extraction parameters and optimize the conditions of phenolic compounds including TPC and TFC from okara, response surface methodology (RSM) with Box–Behnken experimental design (BBD) consisting of seventeen experimental runs was employed.

Since the effects of unexplained variability in the observed response due to extraneous factors could be minimized by randomizing the order of experiments (Silva

et al., 2007), these experiments were conducted randomly to analyze the response pattern and to establish models for total phenolic content and total flavonoid content. The independent variables used in the experimental design were temperature (230-250°C; X_1), residence time (0-10 min; X_2) and liquid-solid ratio (90-110 mL/g; X_3) in three variation levels. Each variable was coded from -1 to 1 so as to affect the response more evenly and render units of the parameters more irrelevant (Baş & Boyacı, 2007).

Table 4-1 Independent variables and their levels used in the response surface design (BBD).

Independent variables	Symbol	Levels		
		-1	0	1
Extraction temperature	X_1	230	240	250
Residence time	X_2	0	5	10
Liquid-solid ratio	X_3	90	100	110

The range and central point value of all the three process variables is shown in Table 4-1. The process variables were coded according to the following equation:

$$x = (X_i - X_0)/\Delta X \quad (4\text{-}1)$$

where x_i is the dimensionless coded value of the independent variable; X_i is the corresponding actual value of the independent variable; X_0 is the actual value of X_i at the central point; and ΔX is the increment of X_i corresponding to a variation of 1 unit of x_i.

After conducting the experiments in replicate, all the data were fitted to the following second order polynomial model (Eq. (4-2)), thereby obtaining the regression coefficients which interpreted the relationship between the responses and the independent variables.

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j + \varepsilon \quad (4\text{-}2)$$

where Y is the predicted response variable associated with each three level combination (TPC, TFC and DPPH), X_i and X_j are the levels of the independent coded variables ($i \neq j$) affecting the response of Y, B_0, B_i, B_{ii} and B_{ij} are the regression coefficients for intercept, linear, quadratic and interaction terms, respectively. The model evaluated the effects of each independent variable on the response. And "ε" is the error.

Analysis of the experimental design and calculation of predicted data was carried out using Design-Expert software to estimate the response of the independent variables and the statistical significance of the terms in the regression equations was examined by ANOVA for each response. Terms statistically regarded as non-significant were excluded from the initial model and the experimental data were re-fitted only to the significant ($p \leq 0.05$) parameters. The optimal conditions of the independent variables were achieved and in turn further applied to the designed model through the same experimental process so that the prediction power of the models could be validated by comparing theoretical predicted data to the experimental data. Triplicate samples of the optimized proportion were prepared and analyzed.

4.2.5 Statistical analysis

Analysis of the experimental design and data was performed using Design-Expert software of version 8.0.5 (Stat-Ease Inc., Min-neapolis, USA). All experimental results were expressed as means ± standard deviations of three measurements (triplicate). All analyses were performed using the Statistical Package for the Social Sciences (SPSS, 19.0) software. A level of $p < 0.05$ was considered to be statistically significant.

4.3 Results and discussion

4.3.1 Determination of the relevant variables and experimental ranges

(1) Effect of extraction temperature on TPC yield

Extraction temperature, residence time and liquid-solid ratio were investigated to determine the appropriately accurate ranges of the designed parameters of TPC extraction yield prior to the further development of response surface models.

As mentioned before, thermal treatment played a pivotal role in the extraction procedure of bioactive compounds by means of affecting ingredient diffusion coefficient, enhancing solvent solubility and improving mass transfer. Consequently, the impact of extraction temperature from 190^{o}C to 250°C on total phenolics extraction level was illustrated in Fig. 4-1a when other two factors, residence time (10 min) and liquid-solid ratio (120 mL/g), were fixed in this study. As shown, a linear relationship was obtained when the temperature increased from the initial one to 240°C, which indicated that extraction temperature had a significantly positive effect on the extraction efficiency and 240^{o}C might be a potential optimum extraction temperature. The TPC yield was 129.27 ± 0.89 mg GAE/g, which as 1.77 times the yield achieved at 190°C. This result is in accordance with other studies (Sani et al., 2014; Zeković et al., 2014), in which TPC yield was claimed to increase dramatically with increasing temperature in a certain range. However, since However, high temperatures are detrimental and a certain upper limit must be considered to avoid degradation of thermo-sensitive phenolics (mainly anthocyanin and flavan-3-ol derivatives) (Escribano-Bailon & Santos-Buelga, 2003), a downward trend exhibited in the later process because of the acceleration of total phenolic content decomposition. Thus, an intermediate temperature of 240°C was adopted for temperature optimization in the Box–Behnken design experiments.

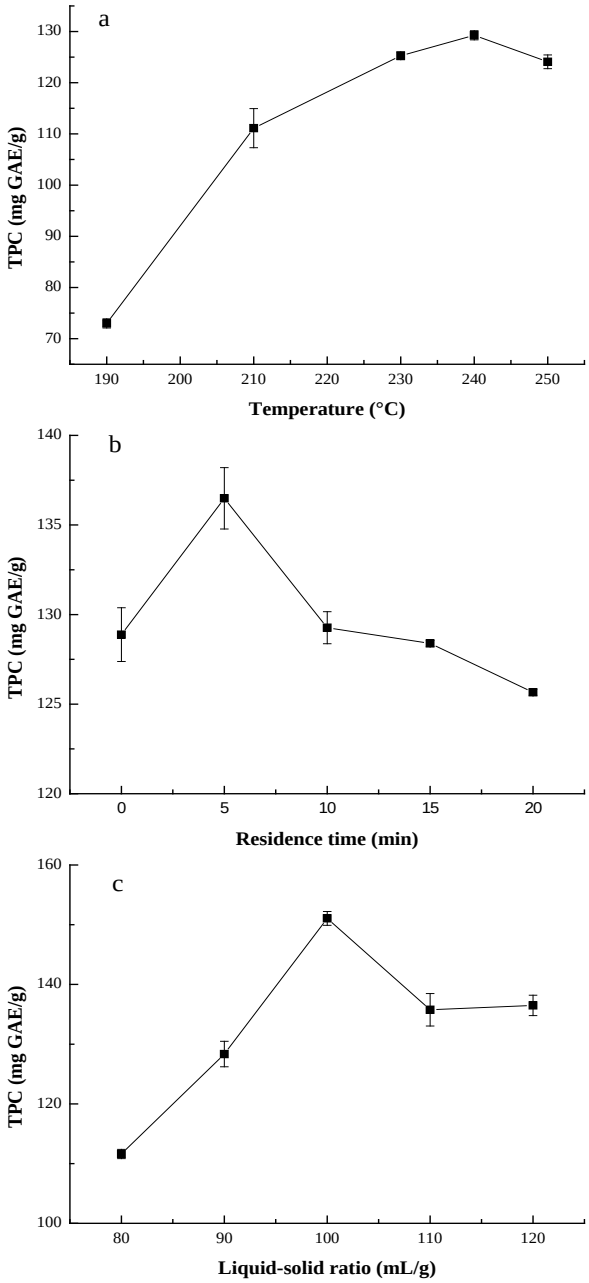


Fig. 4-1 Effect of different temperature (a), extraction time (b), and liquid-solid ratio (c) on the extraction yield of TPC. Data are expressed as means ± standard deviation of triplicate samples.

(2) Effect of residence time on TPC yield

Considering the degradation, energy consumption as well as the extraction efficiency, extractions under different residence time (0, 5, 10, 15, and 20 min) was

conducted when other two parameters, extraction temperature and liquid-solid ratio were fixed at 240°C and 120 mL/g, respectively. Fig. 4-1b showed the effect of residence time on the yield of total phenolics. As shown in this figure, there was an obvious increment from 128.88 ± 1.50 to 136.49 ± 1.71 mg GAE/g during the first 5 minutes, implicating that proper extraction time was beneficial to the extraction process. Nonetheless, it was obviously found that when okara was extracted for 10 min at 240°C, the TPC yield (129.27 ± 0.89 mg GAE/g) was closed to the result that under a residence time of 0 min, suggesting that the aggravation of decomposition had already apparently influenced the yield of the target product. Furthermore, with increase of residence time, a slight decline was still observed, which manifested the rate decomposition had outweighed the extraction rate of TPC. Based on above results, a residence time of 5 min was chosen in the followed-up response surface methodology experiments.

(3) Effect of liquid-solid ratio on TPC yield

Liquid-solid ratio has effects on extraction efficiency as it involves the contact area between raw materials and solvent (Xu et al., 2015). In general, compared with the possibility of incomplete extraction, huge solvent volume contributes to high extraction efficiency due to comprehensive involvement. At the same time, more energy consumption is demanding, which may lead to accumulation of adverse impacts, like the acceleration of hydrolysis, the decomposition of total phenolics and so forth. Therefore, the effect of liquid-solid ratio on extraction yield of TPC was investigated at five ratios (80, 90, 100, 110, and 120 mL/g) under 240°C for a 5 min residence time. The values of TPC were presented in Fig. 4-1c. The result indicated that the extraction efficiency increased significantly when the liquid–solid ratio was increased up to100 mL/g, yielding TPC of 151.06 ± 1.15 mg GAE/g. After that, the extraction yield trend

dropped dramatically at a liquid-solid ratio of 110 mL/g and then decreased graduately. Therefore, the liquid-solid ratio range of 90-110 mL/g was selected as the test range in the followed-up RSM experiments.

In these one-single factor test, three factors, including extraction temperature, residence time and liquid-solid ratio, were investigated to evaluate their influence on the extraction yield of phenolic compounds from okara. Consequently, extraction temperature range of 230-250°C, residence time of 0-10 min, and the liquid-solid ratio range of 90-110 mL/g were employed to optimize the extraction parameters using RSM.

4.3.2 Optimization of SWT using RSM

(1) Model of the responses of the yield of TPC, TFC and DPPH

The results of the predicted and experimentally measured response for the 17 runs of the process optimization according to TPC, TFC and DPPH assay based on Box–Behnken experimental design under different SWT conditions, were summarized in Table 4-2. To optimize the extraction, the central conditions chosen were 100 mL/g liquid-solid ratio at an extraction temperature of 240°C for 5 min. The yield of total phenolic content ranged from 112.76 to 151.79 mg GAE/g on the dry weight basis and the maximum yield was obtained for the 13th run under the experimental conditions of X_1 = 240°C; X_2 = 5 min; X_3 = 100 mL/g. The yield increased significantly from 72.83 to 98.32 mg RE/g on dry weight basis in terms of total flavonoid content, and the maximum yield was obtained as the same run as the highest yield of TPC proceed, which indicated that TFC was the major ingredients of total phenolic content and consistent with the results of previous studies. The DPPH values expressed in millimole (mM) ascorbic acid equivalent (AAE) per 1 g of a sample on a dry weight basis were in the range of 41.03 to 56.18 mM AAE/g dry extract and the extract possessing the

Table 4-2 Combinations of three variables with their coded terms obtained from RSM and observed responses under different experimental conditions.

Run	Temperature (°C) -X_1	Residence time (min) -X_2	Liquid-solid ratio (mL/g) -X_3	TPC (mg GAE/g dry okara)	TFC (mg RE/g dry okara)	DPPH (mM AAE/g dry extract)
1	-1 (230)	-1 (0)	0 (100)	112.764	72.833	41.03
2	1 (250)	-1 (0)	0 (100)	122.114	79.153	44.31
3	-1 (230)	1 (10)	0 (100)	128.130	83.181	45.27
4	1 (250)	1 (10)	0 (100)	120.894	82.347	45.65
5	-1 (230)	0 (5)	-1 (90)	119.927	79.238	44.16
6	1 (250)	0 (5)	-1 (90)	120.073	79.425	44.34
7	-1 (230)	0 (5)	1 (110)	127.528	82.332	45.52
8	1 (250)	0 (5)	1 (110)	127.797	86.381	47.35
9	0 (240)	-1 (0)	-1 (90)	119.195	74.894	42.93
10	0 (240)	1 (10)	-1 (90)	116.707	81.988	47.95
11	0 (240)	-1 (0)	1 (110)	120.550	75.915	46.07
12	0 (240)	1 (10)	1 (110)	127.707	85.617	46.11
13	0 (240)	0 (5)	0 (100)	150.490	97.764	55.98
14	0 (240)	0 (5)	0 (100)	151.789	97.625	55.46
15	0 (240)	0 (5)	0 (100)	149.187	98.319	54.66
16	0 (240)	0 (5)	0 (100)	150.890	96.931	56.18
17	0 (240)	0 (5)	0 (100)	152.033	98.319	55.50

scavenging activity was achieved at 13th run as well. Based on these data, the extraction process was optimized for obtaining desirable responses at maximum.

Table 4-3 Estimated coefficients of the fitted second-order polynomial model for TPC, TFC and DPPH.

Regression coefficient	Response		
	TPC	TFC	DPPH
Intercept			
β_0	150.88	97.79	55.55
Linear			
β_1	0.32	1.22**	0.71*
β_2	2.35**	3.79***	1.33***
β_3	3.46***	1.84***	0.71*
Interaction			
β_{12}	-4.15**	-1.79**	-0.72
β_{13}	0.03	0.97	0.41
β_{23}	2.41*	0.65	-1.25**
Quadratic			
β_{11}	-13.56***	-8.09***	-5.95***
β_{22}	-16.35***	-10.33***	-5.53***
β_{33}	-13.49***	-7.86***	-4.25***

*Significant, $p < 0.05$

**Very significant, $p < 0.01$

***Highly significant, $p < 0.001$

The results of fitting the second-order polynomial model equation (*F*-test) to data and the significance of coefficients (*F* value and *p* value) of the predicted model in coded variables are presented in Table 4-3 and Table 4-4. According to the analysis of variance (ANOVA) on the experimental data, the values of the regression coefficients indicated satisfactory of the model developed. From the ANOVA results of TPC, the regression model of TPC extraction process was of significance as the *F*-value was 139.63 and *p*-value was of a low probability value. The multiple correlation coefficient R^2 was 0.9945, meaning that the sample variation of 99.45% for the TPC extraction yield was statistically significant. But, according to the former study, it could not be

sufficient to ensure that the accuracy and general availability of the polynomial model were satisfactory even though R^2 was nearly equal to one (James et al., 2013). Therefore, *Adj* R^2 (adjusted determination coefficient), as another additional analysis variable for R^2, was applied to render the model more convinced. Results showed that only less than 1.3% of the total variations (*Adj* R^2 = 0.9873) were excluded in this model, signaling the high accuracy of the developed model. Besides, the value of predicted multiple correlation coefficient *Pred.* R^2 (0.9345) was also closed to 1, which further illustrated the correlation between the actual and predicted results was high. The failure of the model to represent the data in the experimental domain at points which are not appearing in the regression was lack-of-fit, whose *F*-value of 3.27 and *p*-value of 0.122 implied that it was not significantly relative to the pure error due to noise. Moreover, the measurement of the signal to noise ratio, Adequate precision, was 29.963, which indicated that the present model could be used to navigate the design space under any combination of values of the variables. Additionally, this model possessed a high degree of precision and a good deal of reliability of the experimental values since coefficient of the variation (C.V.) expressing the standard deviation as a percentage of the mean was 1.22. The levels of significance of diverse coefficients were identified with asterisks in Table 4-4. As a result, the present model could be adequately well for navigating the design space. It could be concluded that one linear coefficient (X_3) and all the quadratic term coefficients (X_1^2, X_2^2, X_3^2) were highly significant with very small *p* values ($p < 0.001$). A linear coefficient (X_2) and one product coefficients (X_1X_2) was very significant ($p < 0.01$). Another cross product coefficient (X_2X_3) was significant ($p < 0.05$). One linear coefficient (X_1) and one cross product coefficient (X_1X_3) were not significant ($p > 0.05$).

Table 4-4 Analysis of variance (ANOVA) of the fitted second-order polynomial model for TPC, TFC and DPPH.

	Sum of squares	DF	Mean square	F-value	*p*-Value
TPC					
Model	3207.8	9	356.42	139.63	< 0.0001
Residual	17.87	7	2.55		
Lack of it	12.69	3	4.23	3.27	0.122
Pure error	5.17	4	1.29		
Cor total	3225.67	16			
C.V. (%)	1.22				
R^2	0.9945				
Adj. R^2	0.9873				
Pred. R^2	0.9345				
Adequate precision	29.963				
TFC					
Model	1269.9	9	141.1	152.19	< 0.0001
Residual	6.49	7	0.93		
Lack of it	5.16	3	1.72	5.19	0.0728
Pure error	1.33	4	0.33		
Cor total	1273.36	16			
C.V. (%)	1.13				
R^2	0.9949				
Adj. R^2	0.9884				
Pred. R^2	0.9337				
Adequate precision	34.136				
DPPH					
Model	425.58	9	47.29	112.30	< 0.0001
Residual	2.95	7	0.42		
Lack of it	1.56	3	0.52	1.50	0.3433
Pure error	1.39	4	0.35		
Cor total	428.53	16			
C.V. (%)	1.35				
R^2	0.9931				
Adj. R^2	0.9843				
Pred. R^2	0.9367				
Adequate precision	28.618				

The three-dimensional and contour plots and the relationships between the independent and dependent variables could be analyzed by the full model as Eq. (4-3).

$$Y_1 = 150.88 + 2.35X_2 + 3.46X_3 - 4.15X_1X_2 + 2.41X_2X_3 - 13.56X_1^2 - 16.35X_2^2 - 13.49X_3^2$$

(4-3)

The quadratic polynomial model of TFC was highly significant and sufficient to represent the actual relationship between the response and significant parameters with very low *p*-value ($p < 0.0001$) from the ANOVA. The model *F*-value (152.19) with an extremely low *p*-value implied that the regression model was significant at 95% confidence level.

The determination coefficients (R^2) of 0.9949 for TFC indicated that the model could significantly explain all the variations. In other words, the closer the quantity of R^2 to1, the better the experimental model fits the real data. However, the smaller the value of R^2, the less relation the dependent variables in the model have in elucidating the behavior of independent variables (Myers et al., 2016). The value of adjusted determination coefficient (*Adj* R^2) 0.9884, was comprehensively proved the model was appropriate and accurate to all the variations. Besides, given the result that predicted multiple correlation coefficient *Pred.* R^2 was 0.9337, it could be indicated that the correlation between the actual and predicted results was significantly high.

The model also showed statistically insignificant lack of fit, as was evident from the computed *F*-values of 0.0728 at 95% confidence level for TFC yield. Furthermore the value of pure error was low, indicating in the good reproducibility of the data obtained with a small p-value from the ANOVA and a satisfactory coefficient of determination.

Adequate precision (34.136) gave an evidence for the present model to navigate the design space under any combination of values of the variables. Moreover, the lesser value of coefficient of the variation (C.V., 1.13) gave a better reproducibility because of the standard deviation as a percentage of mean. Additionally, assorted levels of

significance of diverse coefficients were identified with asterisks in Table 4-4. Overall, the present model could be adequately well for navigating the TFC extraction yield.

As shown, two linear coefficients (X_2, X_3) and all the quadratic term coefficients (X_1^2, X_2^2, X_3^2) were highly significant with very small p values ($p < 0.001$). A linear coefficient (X_1) and one cross product coefficient (X_1X_2) were very significant ($p < 0.01$). Two cross product coefficients (X_1X_3, X_2X_3) were not significant ($p > 0.05$). A full model as Eq. (4-4) was achieved, which can be used to analyze the three-dimensional and contour plots and predict the relationships between the independent and dependent variables.

$$Y_2 = 97.79 + 1.22X_1 + 3.79X_2 + 1.84X_3 - 1.79X_1X_2 - 8.09X_1^2 - 10.33X_2^2 - 7.86X_3^2 \quad (4\text{-}4)$$

Analysis of variance (Table 4-4) demonstrated the relationship between DPPH scavenging ability and significant extraction parameters with an extremely satisfactory regression coefficient (R^2 = 0.9931). Eq. (4-5) shows the mathematical model that described the relationship between the significant independent variables and response of DPPH scavenging activity. According to Fishers F-test, the F value (112.30) of regression coefficient and the corresponding p value (< 0.0001) indicated that the independent variables had a significant effect on the response of DPPH scavenging ability. Also, the ratio of the mean square of lack-of-fit was 1.50 and the p value of 0.3433 corresponding to the lack-of-fit indicated that this one was insignificant and the model is valid. Additionally, various levels of significance of coefficients were marked with asterisks in Table 4-3.

According to the results, one linear coefficient (X_2) and all the quadratic term coefficients (X_1^2, X_2^2, X_3^2) were highly significant with very small p values ($p < 0.001$).

One cross product coefficient (X_2X_3) were very significant ($p < 0.01$). Two linear coefficients (X_1, X_3) were significant ($p < 0.05$). Two cross product coefficient (X_1X_2, X_1X_3) was not significant ($p > 0.05$). A full model as Eq. (4-5) could be applied to analyze the three-dimensional and contour plots as well as predict the relationships between the independent and dependent variables.

$$Y_3 = 55.55 + 0.71X_1 + 1.33X_2 + 0.71X_3 - 1.25X_2X_3 - 5.95X_1^2 - 5.53X_2^2 - 4.25X_3^2 \quad (4\text{-}5)$$

(2) Analysis of response surfaces

As clearly shown in Fig. 4-2A, at the residence time of 0 min, the extraction of TPC increased with extraction temperature until 240°C when liquid-solid ratio was fixed at 100 mL/g. The TPC response started to decrease above this extraction temperature. And a similar increasing trend was observed in the increase in residence time from 0 min to 5 min when extraction temperature and liquid-solid ratio were designed at 230°C and 100 mL/g, respectively. Nevertheless, as it can be seen from Fig. 4-2B, an increase in liquid-solid ratio at 230°C enhanced significantly the TPC extraction when the residence time was fixed at 5 min. The effects of liquid-solid ratio and residence time shown in Fig. 4-2C demonstrated that the TPC value could be superior to 120 mg GAE/g dry matter for a high ratio (liquid to dry matter) and a low level of residence time variable when the extraction temperature was fixed at 240°C.

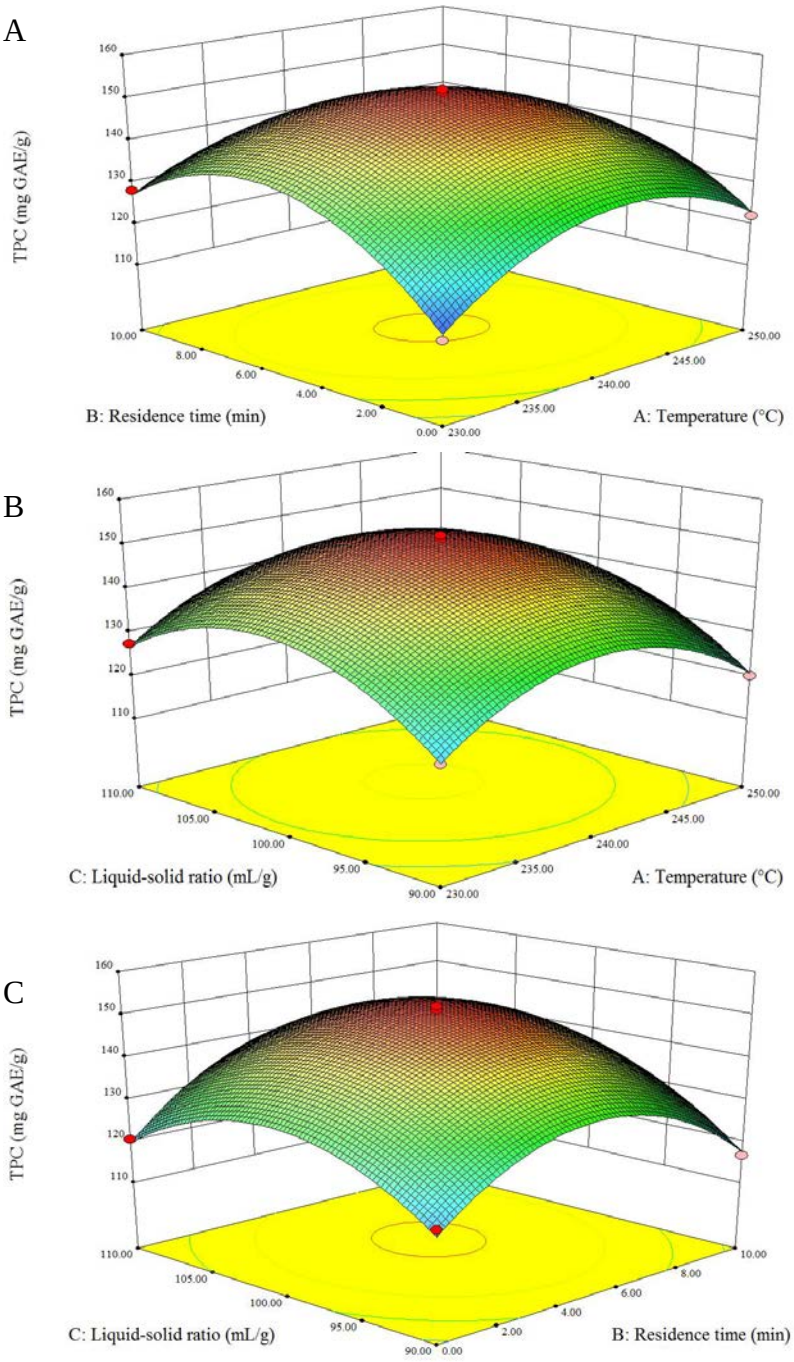
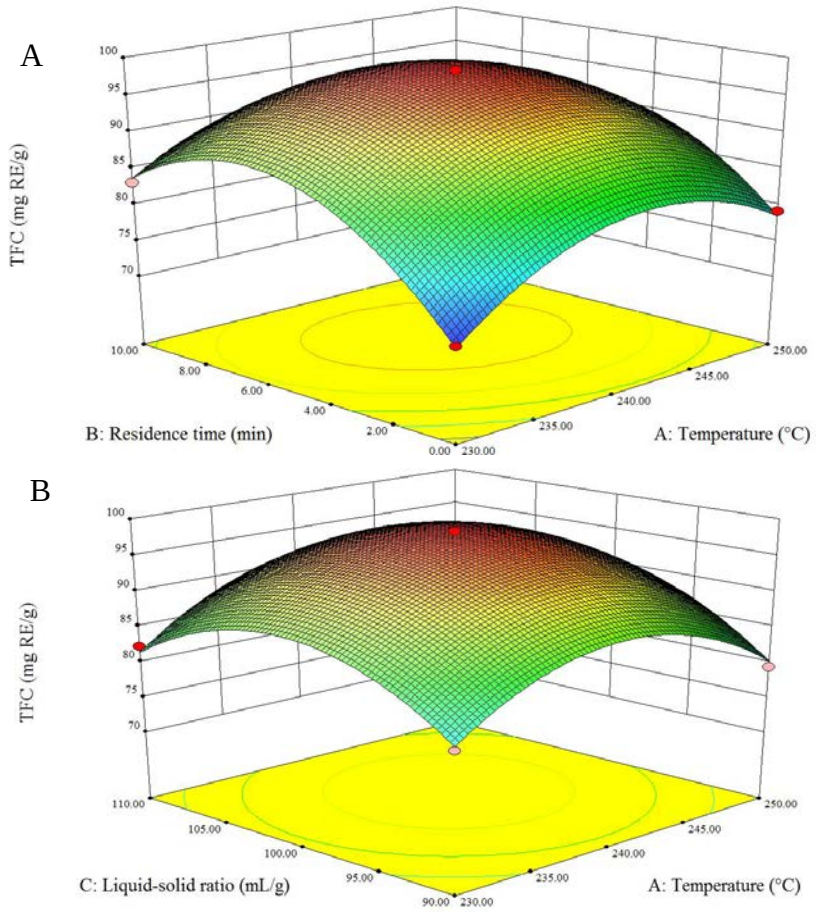


Fig. 4-2 Response surface plots for the effect of (A) temperature and residence time, (B) temperature and liquid-solid ratio and (C) residence time and liquid-solid ratio on the extraction yield of TPC. The value of the missing independent variable in each plot was kept at the centrepoint.

Two and three dimensional response surfaces were plotted for the results of total antioxidant activity in okara extracts using SWT in Fig 4-3. The interaction between all the significant combined factors was exhibited as well. According to Fig. 4-3A, the

increase of both residence time and extraction temperature increased the response of TFC as liquid-solid ratio was fixed at the center. Fig. 4-3B demonstrated that the TFC increased significantly and can surpass the value of 90 mg RE/g with the increase of both extraction temperature and liquid-solid ratio at a fixed residence time of 5 min and then decreased to approximately 80 mg RE/g. It could be seen from Fig. 4-3C that the TFC increased considerably with the increase of liquid-solid ratio and residence time at a fixed extraction temperature of 240°C.



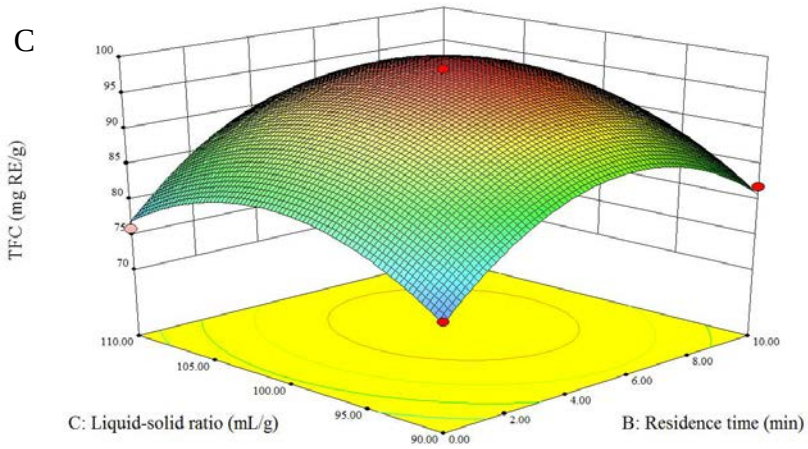


Fig. 4-3 Response surface plots for the effect of (A) temperature and residence time, (B) temperature and liquid-solid ratio and (C) residence time and liquid-solid ratio on the extraction yield of TFC. The value of the missing independent variable in each plot was kept at the centrepoint.

Fig. 4-4 presented the two and three dimensional responses surface of all significant interaction effects of factors. The effect of extraction temperature, residence time and liquid-solid ratio on the DPPH scavenging ability were illustrated. As shown in Fig. 4-4A, when residence time was 5 min, the increase in the antioxidant activity was observed with the increase in extraction temperature until 240^{o}C, and then a decrease in this activity was observed with the increase of extraction temperature. Besides, in Fig. Fig. 4-4B similar linear increase in the DPPH scavenging capacity was observed with the increase in liquid-solid ratio and extraction temperature at a fixed residence time (5 min). Furthermore, the effect of residence time shown in Fig. 4-4C demonstrated that DPPH capacity could be superior to the value of 55 mM AAE/g dry extract at the middle levels of other two variables.

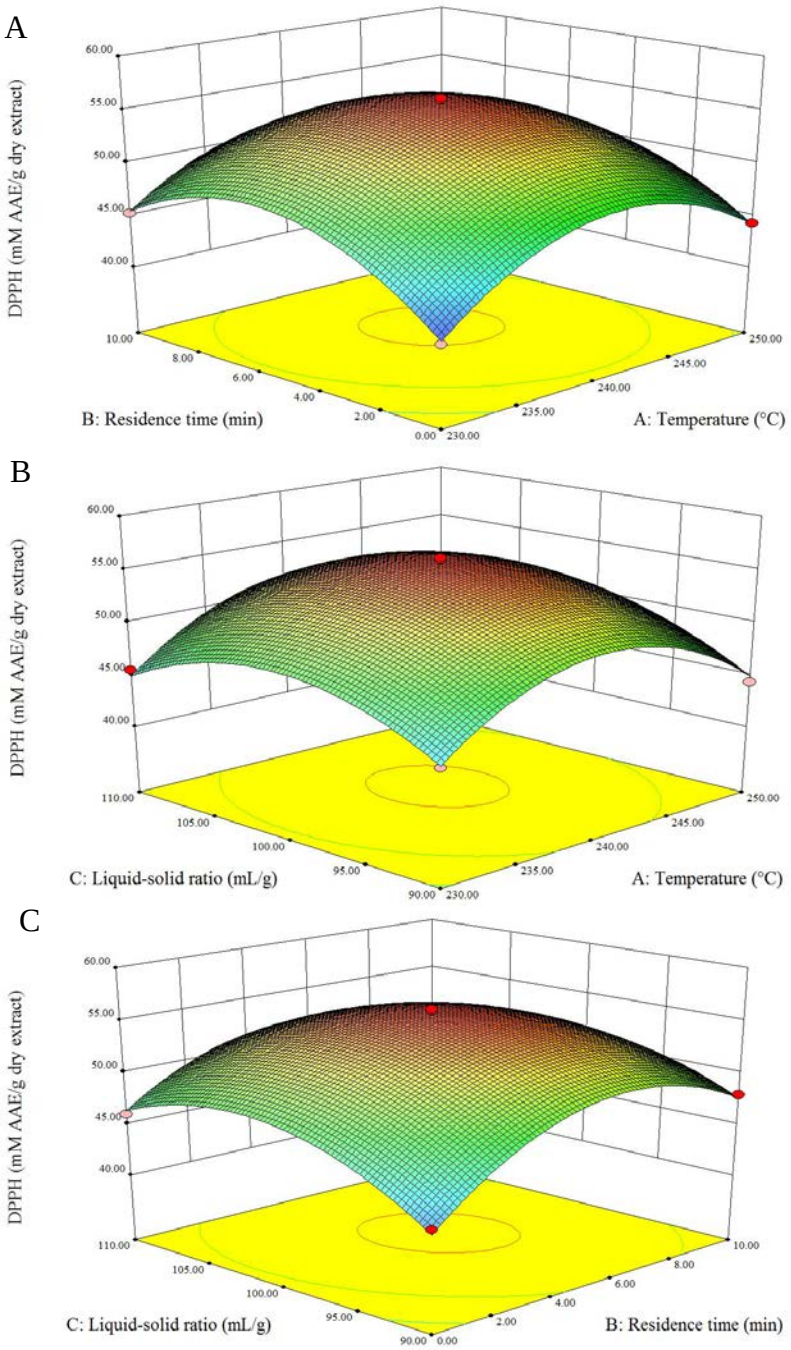


Fig. 4-4 Response surface plots for the effect of (A) temperature and residence time, (B) temperature and liquid-solid ratio and (C) residence time and liquid-solid ratio on the DPPH scavenging activity. The value of the missing independent variable in each plot was kept at the centrepoint.

4.3.3 Optimization of extraction conditions

In order to verify the predictive capacity of the models, optimum conditions were determined using the simple method and the maximum desirability for the maximum

amounts of TPC, TFC and DPPH. The optimum conditions for these three yields were summarized in Table 4-5. According to the results, extraction temperature 240-241°C, residence time in the range of 5-6 min and liquid-solid ratio of 100-102 mL/g, can obtain the optimal TPC (151.21 mg GAE/g), TFC (98.29 mg RE/g) and DPPH (55.66 mM AAE/g dry extract) from okara by using SWT. The desirability function approach of Derringer was used to search the experimental conditions that optimize all the responses simultaneously. Considering the feasible function of the apparatus, the experimental values of TPC, TFC and DPPH at 240°C for 5.7 min with liquid-solid ratio of 101 mL/g were 151.58 ± 0.35 mg GAE/g, 98.35 ± 0.24 mg RE/g and 56.37 mM AAE/g dry extract, respectively. Obviously, the predicted results matched well with the experimental results obtained using optimum extraction conditions which validated the RSM model with a good correlation.

Table 4-5 Estimated optimum conditions, predicted and experimental values of responses under subcritical water conditions.

Response	Extraction conditions			Maximum values	
	Temperature (°C)	Residence time (min)	Liquid-solid ratio (mL/g)	Predicted[a]	Actual[ab]
TPC	239.99	5.41	101.36	151.21	151.58 ± 0.35
TFC	240.63	5.91	101.28	98.29	98.35 ± 0.24
DPPH	240.55	5.54	100.70	55.66	56.37 ± 0.13

[a] The values of TPC, TFC and DPPH were expressed as mg GAE/g, mg RE/g and mM AAE/g dry extract, respectively.

[b] The experimental values of TPC, TFC and DPPH at 240°C for 5.7 min with liquid-solid ratio of 101 mL/g

4.4 Summary

In this work, subcritical water technology was conducted to obtain the extraction of phenolic compounds from okara. The response surface methodology (RSM) using

Box–Behnken design (BBD) was successfully employed to optimize the SWT conditions including three independent parameters (temperature, residence time and liquid-solid ratio) for the achievement of high extraction yield of total phenolic content with high antioxidant activity. The experimental results were adequately fitted with second-order polynomial models and showed significant linear, quadratic and interaction effects of the independent variables. According to the results, extraction temperature 240^{o}C, residence time in the range of 5.7 min and liquid-solid ratio of 101 mL/g, can obtain the optimal TPC, TFC and DPPH from okara by using SWT. All the results showed that okara could be a potential resource for phenolic compounds and SWT appears to have great potential as a method for the extraction of antioxidant materials from foods of plant.

Chapter 5 Conclusions and future research

In this section, previous researches were concisely concluded, and future researches were also prospected.

5.1 Conclusions

In this study, subcritical water technology was employed to extract polysaccharides and phenolic compounds from okara. The main results and conclusions were summarized as follows.

1. SWT was proved to be an efficient and feasible extraction method of achieving polysaccharides from okara. Based on single factor method and response surface methodology with Box–Behnken design, a second-order polynomial equation using multiple regression analysis was analyzed by ANOVA and the optimum processing parameters (temperature of 148^{o}C, residence time of 11.3 min and liquid-solid ratio of 33.0 mL/g) for OP were determined. Under the optimal conditions, the yield of OP increased significantly comparing with that extracted by hot water extraction. Besides, the FT-IR spectrum of OP clearly shows the characteristic functional groups of polysaccharides, and the obtained OP was consisted of some monosaccharides like galactose and arabinose. Furthermore, excellent antioxidant activities of OP using SWT were observed through *in vitro* hydroxyl radical scavenging activity, ABTS scavenging activity and chelating activity, in comparison to that extracted by HWE. The antiproliferative effect of

OP on U2OS cancer cells had been determined without toxicity on TIG-3 normal cells.

2. Thermal processing had positive effects on the phenolic compounds and antioxidant activities of okara extracts in a certain range of temperature (160-230^{o}C). However, total organic content, total sugar and OP decreased under the same circumstance. When other two parameters (residence time of 10 min and liquid-solid ratio of 30 mL/g) were fixed, the highest yields of TPC and TFC were obtained at 220^{o}C. All extracts possessed significantli high scavenging activities on DPPH and ABTS radicals. According to correlation comparisons, the total phenolic contents in the extracts were consistent with the antioxidant activities of the extracts.
3. The response surface methodology (RSM) using Box–Behnken design (BBD) was successfully employed to optimize the SWT conditions including three independent parameters (temperature of 240^{o}C, residence time of 5.7 min and liquid-solid ratio of 101 mL/g) for the achievement of high extraction yield of total phenolic content (TPC), total flavonoid content (TFC) and DPPH assay. The experimental results were adequately fitted with second-order polynomial models which showed significant linear, quadratic and interaction effects of the independent variables.

In consequence, all the results in the present study showed, for the first time, okara could be further developed as a potential antioxidant resource supplying polysaccharides and phenolic compounds extracted by subcritical water technology for

dietary supplements of functional foods. Moreover, subcritical water technology appears to have great potential as a promising technology for the extraction of antioxidant materials from raw materials.

5.2 Future work

1. Although the maximum yield conditions of OP have been determined, isolation and purification of OP should be also concentrated on, thereby yielding specific portions for further utilization.

2. In order to comprehensively identify the thermal effects on chemical constituents of okara extracts using SWT, other ingredients should be introduced in further investigation.

3. Based on the results of antioxidant assays, physiological activities such as anticancer activity, antibiotic activity and other curative effects should be conducted.

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