



Original Article

Possible prebiotics and gallic acid separations from jackfruit seed extract

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Abstract

A large amount of jackfruit seeds can be extracted for possible prebiotics and phenolics (gallic acid) and purified by crystallization and solid phase extraction (SPE), respectively. The studied parameters for crystallization were mixing speed, crystallizing temperature and cooling rate while the studied parameters for SPE were feed concentration, feed flow rate and length to diameter ratio (L/D) of the extraction column. The optimal condition for the crystallization provided the crystal yield of 0.028 g crystal /g dry extract at 100 rpm mixing speed, 58°C crystallizing temperature and 1°C/min cooling rate. Molecular weight analysis of the crystals indicated that the crystals can be fructo-oligosaccharide or raffinose. The optimal condition for SPE obtaining gallic acid 92 percent yield was at 4% w/v feed concentration with 3 mL/min flow rate and L/D of 15.3. Finally a high temperature can degrade both possible prebiotic and gallic acid.

Keyword: prebiotics; gallic acid; jackfruit seeds; crystallization; solid phase extraction

1. Introduction

Recently, functional foods have been gained much interest in health enhancing roles. They are classified in different types, for example, prebiotics and phenolics. Inulin, fructo-oligosaccharide, galacto-oligosaccharide (GOS), xylo-oligosaccharide and raffinose are functional foods and grouped in prebiotics. An anti-oxidant like gallic acid is another type of functional food and classified as phenolics (Campos *et al.*, 2012). Both prebiotics and phenolics can be extracted from vegetables and fruits.

Normally, ripe jackfruits (*Artocarpus heterophyllus* Lam.) are consumed as fresh or processed fruit (canned and snack) (Pua *et al.*, 2010) while a lot of residual seeds are mostly unwanted. A considerable number of researches discovered that jackfruit seeds provides phenolics such as gallic acid (Soong and Barlow, 2004; Junlakan, 2009), possible prebiotics shown in term of non-reducing sugar (Nualla-ong *et al.*, 2009; Wichienchot *et al.*, 2011), reducing sugar and

other impurities. Therefore, the objective of this work was to purify the possible prebiotics and gallic acid from jackfruit seed extract (JSE) by crystallization and solid phase extraction (SPE), respectively.

2. Materials and Methods

2.1 Materials and chemicals

Tongprasert-jackfruit seeds were used in this experiment. Commercial grade ethanol (Lab-Scan Analytical Science, Thailand) was used as a solvent in the extraction of jackfruit seeds. Sulfuric acid (Lab-Scan Analytical Science, Thailand) and phenol (Fisher Scientific, Loughborough, UK) were used in determination of total sugar while 3, 5-dinitrosalicylic acid (Fluka Chemie, Buchs, Switzerland), phenol, sodium potassium tartrate (Ajax Finechem Pty Ltd., NSW, Australia) sodium hydroxide (Lab-Scan Analytical Science, Thailand) and sodium sulfite (Merck, Darmstadt, Germany) were used in determination of reducing sugar. Methanol and hydrochloric acid (Lab-Scan Analytical Science, Thailand) were used in SPE. Folin-Ciocateu reagent (Merck, Darmstadt, Germany) and gallic acid (Sigma-Aldrich, Steinheim, Germany)

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were used as standards. Sodium carbonate was obtained from Ajax Finechem Pty Ltd. (NSW, Australia). D-glucose anhydrous was bought from Sigma-Aldrich (Steinheim, Germany). In addition, C18 Sep-Pak cartridge and C18 were bought from Vertical chromatography Co. Ltd.

2.2 Methods

2.2.1 Preparation of jackfruit extract

The study of possible prebiotic crystallization and gallic acid solid phase extraction required JSE. To prepare JSE, fresh jackfruit seeds were ground (1.0-2.0 mm in size) and then a kilogram of the ground seeds was extracted by 20 L of 1:1 ethanol/water solvent in a batch extractor (Bhornsmithikun, 2010). The condition of extraction was at 90°C for 30 minutes. After that, JSE was filtered by vacuum filter (SIBATA: Circulating Aspirator WJ-20) and the solvent was evaporated by rotary vacuum evaporator (Buchi: Vacuum pump V-700) until its concentration was about 0.4 g/mL. Then JSE was freeze-dried to get crude dry extract (Nualla-ong *et al.*, 2009). This gave six grams of dry extracts per kilogram of the ground seeds.

2.2.2 Possible prebiotic crystallization (crystallizing procedure and conditions)

Firstly, a range of crystallizing temperature of a dry JSE was determined by differential scanning calorimeter (DSC). Afterward, JSE solution (concentration 0.4 g/mL and viscosity 15-16 cp) was heated to 85°C for 10 minutes and then cooled down to the new desired crystallizing temperature range from DSC. The cooling rate of 0.5, 1.0, 1.5 and 2.0°C/min and mixing speeds of 0, 50, 100, and 150 rpm (Reynolds number 5,000-14,400) which provided turbulent mixing were the studied conditions for the crystallizing process. The effects of these parameters on the crystal yield and amount of the possible prebiotic in terms of non-reducing sugar were considered. Non-reducing sugar (NRS) was examined from total sugar (TS) and reducing sugar (RS) as shown by $NRS = TS - RS$. Methods used to determine total sugar and reducing sugar were the modified phenol sulfuric method (Bubois *et al.*, 1956) and the modified dinitrosalicylic acid (DNS) method (Miller, 1959), respectively. The available method to analyze the molecular weight of the JSE before and after crystallization was gel permeation chromatography (GPC) examined by the Material Technology Center, a division of the National Science and Technology Development Agency, Bangkok, Thailand. The samples for GPC analysis were dissolved in 0.1 M $NaNO_3$ before filtrate and the sampling of 20 μ L were injected into the GPC column with RI detector at flow rate of 0.6 mL/min and temperature 30°C as stated by Wichienchot *et al.* (2011)

2.2.3 Gallic acid extraction (purification procedure and conditions)

1) Design of extraction conditions

For gallic acid extraction, the designed conditions were divided into two parts. The first part focused on the optimal feed concentration of crude JSE fed into C18 Sep-Pak cartridge. The other part focused on the process in C18 packed column which the effect of volumetric flow rate and length to column diameter ratio of C18 column (L/D) were considered.

Optimal feed concentration of crude JSE used in C18 Sep-Pak cartridges

The studies of optimal feed concentrations of JSE before and after crystallization were done in C18 Sep-Pak cartridge as shown in Figure 1. After preconditioning step, JSE with designed concentrations of 3, 4, or 5 % w/v was loaded into the cartridge. The viscosity of each JSE concentration was measured by viscometer. Next, the cartridge was left at room temperature for 1 hour before washing step and eluting step (Li *et al.*, 2006). The composition of the loaded JSE and the elute JSE were analyzed by HPLC (Agilent 1100 Series HPLC) with Zorbax Eclipse XDB C8, 4.6 x 250 mm column. The condition for HPLC was at wavelength detector 280 nm for gallic acid and 30°C column temperature. The mobile phase consisting of acetonitrile and 1% acetic acid (55:45 v/v) maintained the flow rate at 0.8 mL (Junlakan, 2009).

Gallic acid purification in C18 packed column

The proper concentration from the above was used in an upscale C18 packed column. The volumetric flow rates of crude JSE from 2 to 5 mL/min and L/D ratio of C18 packed column from 2.2 to 40.5 (with a constant weight of C18, 12 g)

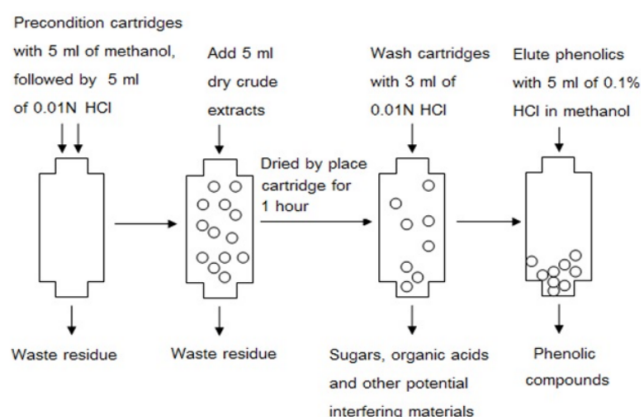


Figure 1. Extraction of gallic acid from crude jackfruit seed extract using C18 (Li *et al.*, 2006).

were the studied parameters. After dilution, gallic acid was analyzed by HPLC as previously mentioned.

2) Purification of eluted gallic acid

This part focused on the effect of neutralization and evaporation of solvents (HCL and methanol) from the eluted gallic acid on the amount of gallic acid. The elute JSE in C18 Sep-Pak cartridges with the feed concentration of 4% w/v was used in this study. After the elution, gallic acid needed to be purified from the mixtures of gallic acid, methanol and HCl by neutralizing HCl and evaporating methanol. There were three cases of the examination: A) Evaporate methanol at the room temperature and then neutralize HCl by 0.1% w/v NaOH, B) Evaporate methanol at 50°C for two hours and then neutralize HCl by 0.1% w/v NaOH and C) Only neutralize HCl by NaOH. The amount of eluted gallic acid before and after purification steps were analyzed by HPLC.

3) Efficacy of C18 regeneration

For the economic consideration, the study of reused C18 was done. C18 in the packed column was regenerated by methanol after the elution of gallic acid. The conditions of the extraction in this part were 3 mL/min and 4%w/v of JSE in 15.3 L/D packed column. The yield of gallic acid from using new C18 was compared with the yield from using regenerated C18.

2.2.4 Possible prebiotics and gallic acid separation sequence

Since the possible prebiotic and gallic acid can be extracted from JSE, therefore the sequence of the possible prebiotics and gallic acid separations can be considered in two sequences: the first sequence was the crystallization of the possible prebiotics before the gallic acid extraction and the second sequence was the gallic acid extraction before the possible prebiotic crystallization. Both sequences operated at the optimal conditions of each separation from the previous parts.

2.3 Statistic analysis

Analysis of variance (One-way ANOVA) and Duncan's multiple range tests at 95 percent confident was used in this study.

3. Results and Discussion

3.1 Optimal condition for possible prebiotics crystallization

By DSC, the melting point and the crystallizing point of dry JSE were 63.7°C and 55.3°C, respectively as shown in Figure 2. Therefore, this work studied the crystallizing temperature from 55°C to 64°C. The results of mixing speed, crystallizing temperature and cooling rate of crystallization

were shown in Table 1.

3.1.1 Effect of mixing speed

From Table 1, the amounts of the crystal and the non-reducing sugar per dry extract increased when increasing mixing speed from 0-100 rpm which the Reynolds number varied from 0 to 9,600 because agitation supported well mixing and provided nucleus formation (Taylor 1973; Berger, 1977; Chetpattananondh and Tongurai, 2008). However, the mixing speed at 150 rpm created low crystallization since too high mixing speed properly decomposed nucleus formation (Taylor, 1973; Berger, 1977; Chetpattananondh and Tongurai, 2008). Additionally, the mixing speed did not effect on the amount of total sugar and non-reducing sugar of the crystal as shown in column 5-7 in Table 1 because of no transformation to other molecules for the same operating temperature (Matusek *et al.*, 2009).

3.1.2 Effect of crystallizing temperature

The crystallizing temperature insignificantly affected the crystal yield and amount of total sugar as shown in Table 1. However, the crystallizing temperature affected the amount of reducing sugar and non-reducing sugar. The higher temperature decreased the amounts of reducing sugar and non-reducing sugar because of the molecular degradation (Matusek *et al.*, 2009). The temperature which gave the highest amount of the crystal (0.026 g crystal/g dry extract) was 58°C. Thereby, the best temperature for the crystallization was 58°C.

3.1.3 Effect of cooling rate on crystallization

From Table 1, crystallization at high cooling rate (about 1.5-2°C /min) caused a lower amount of the crystal formation because of the shorter time of crystallization. The cooling rate of 1°C/min gave the highest amount of the

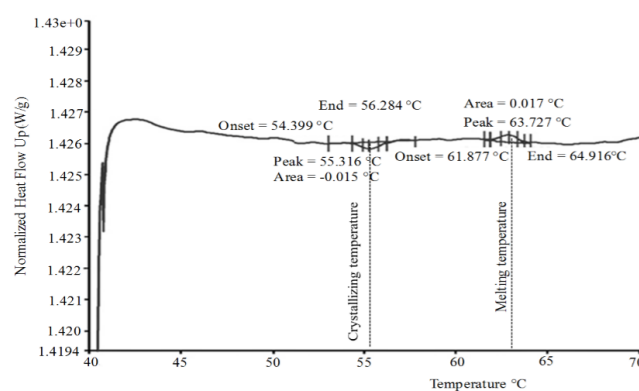


Figure 2. Analysis of crystallizing temperature and melting temperature of dry jackfruit seed extract by differential scanning calorimeter at 61°C.

Table 1. Effect of mixing speed, crystallizing temperature and cooling rate on crystallization of jackfruit seed extract at concentration 0.4 g/mL.

Mixing speed (rpm)	Crystallizing temperature (°C)	Rate of cooling (°C/min)	Yield of crystal (g / g dry extract)	Total sugar (mg Glu / g crystal)	Reducing sugar (mg Glu / g crystal)	Non-reducing sugar (mg Glu / g crystal)	Non-reducing sugar (mg Glu / g dry extract)
0			0.019 ^a	863.8±1.04 ^a	385.4±1.83 ^a	478.43±1.11 ^a	9.2±0.21 ^a
50	61	1.0	0.024 ^c	864.4±2.04 ^a	386.8±2.62 ^a	477.6±1.09 ^a	11.8±0.24 ^c
100			0.026 ^d	865.0±1.92 ^a	386.3±1.91 ^a	478.6±1.84 ^a	12.5±0.48 ^d
150			0.023 ^b	865.1±1.92 ^a	387.9±1.47 ^a	477.1±1.84 ^a	11.0±0.04 ^b
100	55		0.025 ^{ab}	881.8±0.58 ^{ab}	383.6±1.19 ^d	498.2±1.31 ^c	12.5±0.26 ^{bc}
	58	1.0	0.026 ^c	873.83±1.58 ^c	379.6±0.66 ^c	494.2±1.99 ^b	12.7±0.68 ^c
	61		0.025 ^{bc}	860.83±1.52 ^{bc}	377.4±1.45 ^b	483.3±2.90 ^b	12.3±0.74 ^b
	64		0.024 ^a	846.61±3.10 ^a	372.4±1.31 ^a	474.2±4.35 ^a	11.8±0.02 ^a
100	58	0.5	0.024 ^b	875.5±0.67 ^a	378.0±0.25 ^b	497.5±0.86 ^b	12.2±0.18 ^b
		1.0	0.027 ^d	875.6±0.82 ^a	374.5±0.41 ^a	501.0±0.45 ^b	13.7±0.01 ^d
		1.5	0.026 ^c	875.4±0.42 ^a	375.7±2.34 ^{ab}	499.6±2.23 ^{ab}	12.9±0.13 ^c
		2.0	0.022 ^a	876.4±0.91 ^a	376.2±0.26 ^{ab}	500.2±0.72 ^b	10.8±0.10 ^a

Note: Results are presented as means ± S.D. for triplicate analyses; the same letters indicate that the value is not different significantly by the means of One-Way ANOVA and Duncan's Multiple Range Test at 95 percent confident.

crystal, 0.027 g crystal/g dry extract. However, based on gram of the crystal, cooling rate did not affect the amount of total sugar and reducing sugar as shown in column 3 of Table 1 because the molecules did not transform to others for the same temperature (Matusek *et al.*, 2009) as the same reason as the effect of the mixing speed. Thus, based on gram of dry extract, the cooling rate of 1°C/min gave the highest amount of non-reducing sugar (13.7 mg glucose/g dry extract).

Therefore, the optimal condition for the possible prebiotic crystallization was the cooling rate of 1°C/min at 58°C of crystallizing temperature and 100 rpm of mixing speed. This condition provided the crystal yield of 0.027 g crystal/g dry extract and achieved non reducing sugar 13.70 ±0.01 mg glucose/g dry extract. Increasing possible prebiotic purity can be done by two-step or higher crystallization (Chetpattananondh and Tongurai, 2008).

3.1.4 Molecular weight of the crystals

Molecular weights of the crystals analyzed by gel permeation chromatography (GPC) were shown in Figure 3 to 5. The main components in JSE before crystallization were monosaccharide and disaccharide (molecular weight ~ 246 Dalton or degree of polymerization (DP) ~ 1 to 2), some middle size of sugar (~ 883 Dalton or DP: 5) and a few of high molecular weight sugar (~1367 Dalton or DP: 7) as shown in Figure 3. As a result, this JSE contained oligosaccharide, feasibly, prebiotics, which had DP 3 to 9 (Cumming and Staphen, 2007; Wichienchot and Hatupornpipat, 2009). The remaining sugars after crystallization had DP 1-3 as exposed in Figure 4. It was found that the crystals contained the

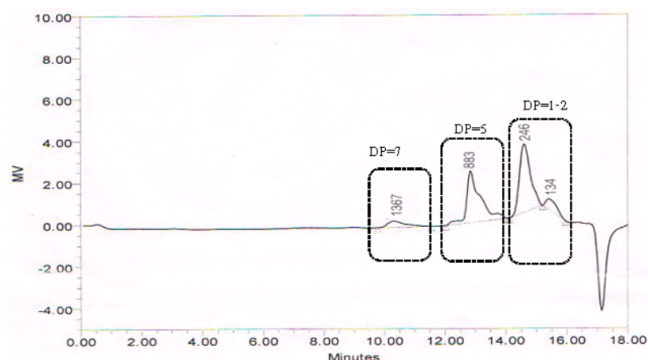


Figure 3. Gel permeation chromatogram of jackfruit seed extract before crystallization.

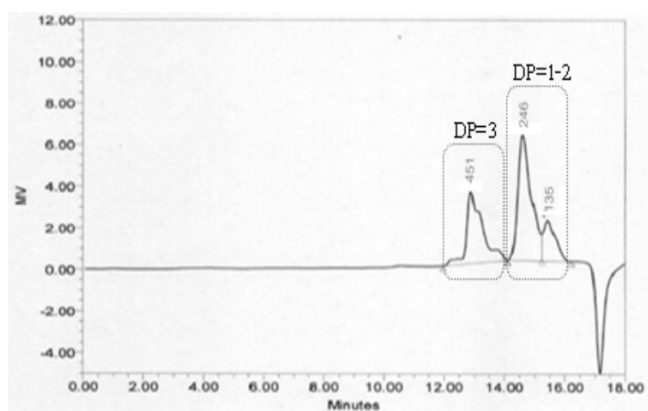


Figure 4. Gel permeation chromatogram of jackfruit seed extract after crystallization at 58°C, 100 rpm and cooling rate 1°C/min (remove crystals).

matters which molecular weights were about 1367 Dalton or DP 7 as presented in Figure 5. These molecular weights were close to the molecular weights of fructo-oligosaccharide (1-ketose, 6-ketose and neoketose) (Wichienhot *et al.*, 2009; Benkeblia, 2013) or raffinose (Gilbert, 1997).

3.2 Optimal condition for gallic acid separation by SPE

3.2.1 Effect of feed concentration for C18 Sep-Pak cartridges

Table 2 expressed the effect of extract concentration on % yield and the amount of gallic acid. The amount of gallic acid contained in elutes from the C18 Sep-Pak cartridges indicated the optimum feed concentration in this study was 4%w/v for both JSE before and after crystallization. The

lower concentration of JSE had a smaller amount of gallic and lower yield as the same result of Pinelo *et al.* (2005). On the other hand, a higher concentration (gallic acid and some impurities) had a higher solution viscosity (viscosity of crude extracts at the concentration of 3, 4 and 5 %w/v were 1.06, 1.24 and 1.43 cp, respectively) and a greater amount of impurities to be absorbed by sorbent.

3.2.2 Effect of volumetric feed flow rate on phenolics extraction

The optimal feed concentration of 4%w/v was used in the study of feed flow rate. From Table 3, increasing flow rate from 2 mL/min to 3 mL/min increased yield of gallic acid as a result of a higher flow rate yielded a higher amount of

Table 2. Effect of jackfruit seed extract feed concentration through the C18 Sep-Pak cartridges before and after crystallization on amount of gallic acid.

Concentration (%w/v)		Amount of gallic acid (mg Gallic acid/ g dry extract)		% Yield
		Before loading to C18	Elute from C18	
Jackfruit extract before crystallization	3	2.26±0.03d	1.63±0.02a	72.46a
	4	2.22±0.01d	1.92±0.03c	86.49c1.
	5	2.24±0.02d	1.85±0.01b	82.79b
Jackfruit extract after crystallization	3	1.80±0.03d	1.32±0.03a	73.06a
	4	1.79±0.02d	1.51±0.03c	83.93b
	5	1.82±0.02d	1.44±0.02b	79.21b

Note: Results are presented as means ± S.D. for triplicate analyses; the same letters indicate that the value is not different significantly by the means of One-Way ANOVA and Duncan's Multiple Range Test at 95 percent confident.

Table 3. Effect of feed flow rate and length to diameter ratio (L/D) of C18 packed column on % yield of gallic acid at feed concentration 4% w/v.

Conditions		% Yield of gallic acid
Volumetric Feed flow rate (ml/min)	L/D	
2	2.2	73.85c
3		78.71d
4		62.03b
5		54.98a
3	2.2	78.71d
15.3	90.22c	
40.5	84.41b	

Note: For triplicate analyses; the same letters indicate that the value is not different significantly by the means of One-Way ANOVA and Duncan's Multiple Range Test at 95 percent confident.

entered gallic acid. However, at flow rate greater than 3 mL/min, gallic acid decreased because of a more turbulent condition which decreased a thickness of the solution around a sorbent and caused a shorter time for the sorbent to extract the solution. As the result, the optimal feed flow rate was 3 mL/min at the feed concentration of 4% w/v.

3.2.3 Effect of L/D of packed C18 on gallic acid extraction

With the optimal feed concentration and feed flow rate for the same amount of packed C18, the effect of L/D of C18 packed column was also exposed in Table 3. L/D ratio of 15.3 gave the highest percent yield of 90.22 because a wider diameter caused a shorter distance of extraction which gave a smaller diving force. Then, less solution was extracted whereas a shorter diameter and a longer length took a more time for adsorption and desorption.

3.2.4 Effect of methanol evaporation and neutralization on amount of gallic acid in gallic acid purification

From Table 4, the methanol evaporation at room temperature ($30^{\circ}\text{C} \pm 2^{\circ}\text{C}$) and only neutralization did not effect on the amount of gallic acids (81.05 mg Gallic acid/ L JSE and 80.31 mg Gallic acid/ L JSE, respectively) whereas a higher temperature of evaporation (50°C) reduced amount of gallic acid as Boles (1988) found that gallic acid in aqueous solution speedily decomposes at temperatures between 105 and 150°C . Thus, the gallic acid purification should be done at room temperature for methanol evaporation before or after neutralization.

3.2.5 Efficacy of C18 regeneration

At 3 mL/min feed rate and 4%w/v of JSE in 15.3 L/D packed column, the extraction by using new C18 pack column gained 90.22 percent yield of gallic acid while the regenerated C18 column gained 22.91 percent yield of gallic acid. It can be seen that an efficiency of using regenerated

C18 extraction was about 77% lower than using the new one. As a result, other regeneration methods should be studied e.g. using other solvents or more washing.

3.3 Effect of prebiotics and phenolics separation sequence

To study the separation sequence, the optimal condition for crystallization ($1^{\circ}\text{C}/\text{min}$ of cooling rate, 58°C of crystallizing temperature and 100 rpm of mixing speed) and the optimal condition for SPE in C 18 packed column (4 % w/v of concentration, 3 mL/min of feed flow rate and L/D 15.3) were used. From the study found that crystallization before extraction (sequence I) gave a non-reducing sugar 14.05 ± 0.09 mg Glucose/ g dried extract while extraction before crystallization (sequence II) gave 13.36 ± 0.19 mg Glucose/ g dried extract since some non-reducing sugar was extracted by C18 (Li *et al.*, 2006; Irakli *et al.*, 2012). For gallic acid extraction, Sequence II provided a gallic acid of 2.21 ± 0.02 mg gallic acid/ g dried extract whereas sequence I produced 1.35 ± 0.01 mg gallic acid/g dried extract because heat from crystallization could alter the molecules (Matusek *et al.*, 2009). As a result, the sequence of the separation depended on the desired product. The most valuable one should be firstly separated.

4. Conclusions

The optimum condition of possible prebiotics crystallization occurring at mixing speed of 100 rpm, crystallization temperature of 58°C and cooling rate of $1^{\circ}\text{C}/\text{min}$ provided the crystal yield of 0.028 g crystal /g dry extract and achieved 14.05 ± 0.09 mg glucose/g dry extract. The crystals from JSE were feasibly fructo-oligosaccharide or raffinose. For C18 solid phase extraction, the optimal operating condition gave percent yield of gallic acid 90.22 at 15.3L/D ratio of packed column for concentration of JSE 4%w/v and flow rate of 3 ml/min. In addition, a high temperature can degrade both possible prebiotic and gallic acid. Finally, the better sequence of separation depends on the value or desired product.

Table 4. Effect of methanol evaporation and neutralization of the elute from C18-Sep-Pak Cartridges with the concentration of 4% w/v jackfruit seed extract feed on the amount of gallic acid.

Conditions	Amount of gallic acid (mg Gallic acid/ L JSE)
Control (JSE after SPE)	$82.73 \pm 0.87\text{b}$
Room temperature ($30 \pm 2^{\circ}\text{C}$) evaporation and neutralization	$81.05 \pm 0.10\text{b}$
50°C evaporation and neutralization	$62.21 \pm 0.18\text{a}$
Neutralization	$80.31 \pm 2.39\text{b}$

Note: For triplicate analyses; the same letters indicate that the value is not different significantly by the means of One-Way ANOVA and Duncan's Multiple Range Test at 95 percent confident.

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