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Potential Use of Cross-flow Microfiltration System in Separation of Functional Compound from Fermented Beetroot (*Beta vulgaris L.*) as Natural Oxidation Prevention

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Abstract*— This study was conducted to determine the potential utilization of microfiltration (MF) membrane in separating functional compounds from beetroot (*Beta vulgaris* L.) biomass as a functional drink for natural oxidation prevention. Separation was performed through MF membrane (pore size of 0.15 µm) at room temperature, flow rate ~7.5 L/min, and TMP 2 and 6 bar for 0, 5, 15, 25, and 35 minutes. The results showed that process optimization based on gallic acid as total polyphenols and acetic acid were achieved at TMP 2 and 6 bar for 35 minutes, respectively. At TMP 2 and 6 bar produced retentate with acetic acids 1.24 and 0.95%, gallic acid 0.42 and 0.41%, total solids 3.49 and 3.47%, total sugars 36.64 and 44.66 mg/mL, pH 3.11 and 3.10, and inhibiting ability of 62.45 and 58.48%, respectively, meanwhile permeate had acetic acid 0.73 and 0.82%, gallic acid 0.31 and 0.33%, total solids 3.39 and 3.38%, total sugars 40.95 and 61.56 mg/mL, pH 3.12 and 3.13, inhibiting ability of 47.62 and 52.43%, respectively. In these conditions, CF-MF is technically able to retain acetic acid (2.63-folds) and gallic acid (1.21-folds) in the retentate and increase inhibition by 25.12 and 11.25% in comparison with the initial process (0 minutes). The LC-MS analysis of permeate at TMP 2 and 6 bar for 35 minutes were predominated by monomers of acetic acid and gallic acid with MW 61.2450 Da. (M+) and 193.0327 Da. (M+Na+) and relative intensities 100 %.

***Keywords*— Beetroot; gallic acid; acetic acid; inhibition; cross-flow microfiltration (CF-MF).**

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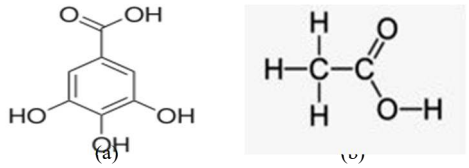
I. INTRODUCTION

A. Background

Gallic acid and acetic acid present in fermented beetroot (*Beta vulgaris L.*) has the potential as an alternative bioactive in functional drinks for preventing natural oxidation. Beetroot biomass yielded polyphenol and acetic acid during fermentation by kombucha culture, particularly *Acetobacter sp.*[1] Furthermore, it pertained to anticholesterol activity [2]. Polyphenol can prevent cells from exposure to free radicals due to oxidation reaction [3, 4] followed by oxidative stress [5]. Thus, it may lead to degenerative illness [5, 6]. Meanwhile, acetic acids decrease blood cholesterol by converting cholesterol into coprostanol, which lowers cholesterol absorbed by the body [7, 8]. Fig. 1a and 1b illustrate the chemical structure of gallic acid and acetic acid.



(a) (b)

Fig. 1 Chemical structure of (a) gallic acid and (b) acetic acid.

Acetic acid (CH3–COOH) is a compound resulting from a biological process, which is a colorless liquid with an aroma like vinegar and has a molecular weight (MW) of 60.052 g/mole, a density of 1.05 g/cm³, the boiling point of 117.9 °C, melting point 16.6 °C. Currently, acetic acid is used as an acidity controller, water softener, and food additive in the food industry and household industry [9, 10].

Gallic acid (GA) (3,4,5-trihydroxy benzoic acid, $C_7H_6O_5$ is a white anhydride crystal, yellowish-white crystal, or pale yellowish-brown crystal with a molecular weight (MW) of 170.12 g/mole. GA is a monomeric phenolic compound solid and colorless, bound to sugars, and belongs to a group of

hydrolysable tannins. GA can regulate several biological activities, such as anti-inflammatory, antibacterial, antioxidant, cardioprotective, antiviral, and anticancer [11, 12, 13, 14].

The separation process of acetic acid and gallic acid from fermented beetroot through the CF-MF system is performed and results in concentration and permeation in the module scale (feed volume of 9 L). Coagulation/flocculation (CF) and microfiltration (MF) processes, as well as the combination of both processes (CF-MF) were conducted[15, 16]. CF-MF process by MW and particle size difference can distinguish particles with sizes larger than 0.1 – 10 μm at TMP between 0.3 and 3.3 bar. During the process, compounds with particle sizes from 0.1 to 10 μm will pass freely as permeate (pigment, organic acids, amino acid, vitamin, and mineral) [17, 18], apart from the occurrence of fouling, which is affected by pressure and time of the process, type of membrane, and characteristic of biomass [19, 20]. In the CF-MF system, the feed flows parallelly (tangentially) on the membrane surface, creating shear force to minimize fouling and avoid the occurrence of concentration polarization (CP), and facilitate concentrate or retentate flow to the feed tank and mix it as new feed[21]. By differentiating the pressure and time of the purification process, the optimum purification level can be achieved. Liquid Chromatography identified characteristics of acetic acid and gallic acid with Mass Spectrometry (LC-MS) based on their molecular weights (MW)[22].

B. Objective

This study aims to determine the optimum process of separating functional compounds from fermented beetroot by CF-MF system at fixed conditions (room temperature, flow rate ~7.5 L/min.) on physicochemical composition, particularly gallic acid and acetic acid. The characteristic of gallic acid and acetic acid monomers and inhibiting ability of biomass before and after the purification process as functional compounds for preventing natural oxidation was also observed.

II. Material and Methods

A. Materials

The main materials used in this experimental work were fresh beetroot (Beta vulgaris L.) and sucrose (local market), Kombucha culture (Research Center for Chemistry – LIPI), commercial MF membrane with a pore size of 0.15 μm (Alfa Laval, Nakskov, Denmark), and all chemicals used in the analysis of total solids, acetic acids, total polyphenol, and total sugars, pH, and inhibiting ability were of analytical grade.

B. Equipment

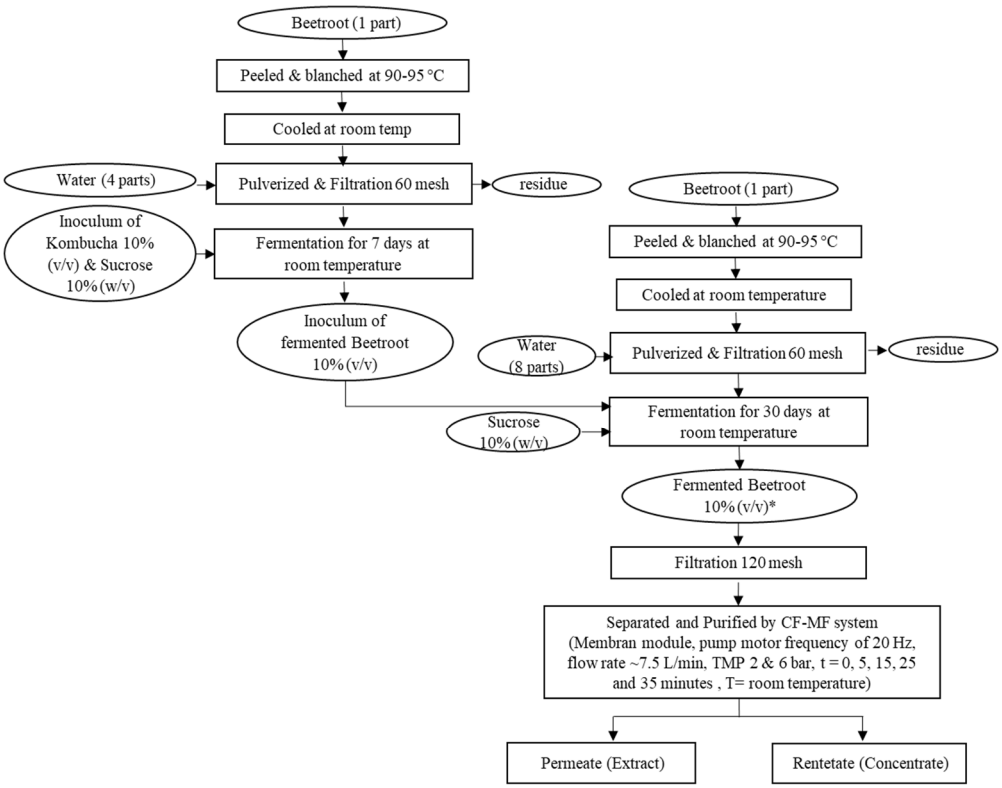
Fermentation systems (autoclave, laminar airflow, incubator), cross-flow membrane filtration module system of DDS LabUnit M20 with both adjustable membrane area and operational pressure (Danish Separation Systems AS, Nakskov, Denmark) [23] and analysis instrument including UV-Vis Spectrophotometer (Model RF-550, Shimadzu, Japan), Liquid Chromatography tandem Mass Spectrometry (Mariner Biospectrometry) installed with LC (Hitachi L 6200) were used in this study.

C. Procedure

1) Experimental design: This study was conducted using fermented beetroot and concentrated through CF-MF system at room temperature, a fixed pump motor frequency of 20 Hz (tangential flow rate of ~7.50 L/min.), and TMP 2 and 6 bar for 0, 5, 15, 25, and 35 minutes. Analysis was performed on total solids (Gravimetric), acetic acid (Titration), total sugars (Phenol Sulphate), and gallic acid as total polyphenol (Denise-Folin)[24]. Sample at optimum condition was determined for inhibition and identified for acetic acid and gallic acid using LC-MS. Process and analysis were carried out in duplicate. Data were processed in descriptive analysis based on the average value.

2) The fermentation process of beetroot (Beta vulgaris L.) in semi pilot scale: Fermented beetroot in a column was made by pulverizing one (1) part of blanched beetroot and four parts of pasteurized water at 90 – 95 °C, then cooled and added with sucrose 10% (w/v, filtrate), inoculated with Kombucha culture 10% (v/v, filtrate), and stored in a closed container for seven days. Next, the inoculum filtrate was sieved through 60 mesh and obtained for semi-pilot fermentation. Fermentation in semi pilot scale (7 L) was performed with a similar initial step. However, the pulverization was prepared by mixing one (1) part of blanched beetroot and eight parts of pasteurized water at 90 – 95 °C, added with sucrose 10% (w/v, filtrate of beetroot) and beetroot inoculum 10% (v/v, filtrate of beetroot). Fermentation was carried out in a closed container at room temperature for 30 days. Then, recovered biomass was sieved through 120 mesh. The resulted in suspension as the initial material was separated and purified through CF-MF system.

3) Separation of fermented beetroot biomass by CF-MF: The feed tank of MF module was filled with 3 L of fermented beetroot suspension to about one-third of capacity (9 L). The fermented beetroot suspension was pumped through SS-filter 200 μm into the membrane module at room temperature (flow rate ~7.5 L/min, TMP 6 bar) for about 5 minutes until the permeate contained clear red liquid and retentate (concentrate), during which both permeate and retentate (concentrate) were returned to the feed tank. The permeate and retentate (concentrate) samples were collected and stored in clean glass bottles and permeate flow rate passing through the membrane was sampled for 0, 5, 15, 25, and 35 minutes, respectively. On the following MF process, the TMP was changed to 2 bars for 0, 5, 15, 25, and 35 minutes at room temperature and flow rate ~7.5 L/min, respectively. All samples of permeate and retentate (concentrate) were kept in the freezer to prevent deterioration. The samples were then analyzed for total solids, total sugars, acetic acids, gallic acids, and inhibiting ability



* Fermentation in semi pilot scale (7 L)

Fig. 2 Fermentation process of beetroot (*Beta vulgaris* L.) in semi pilot scale and Separation of fermented beetroot biomass by CF-MF

III. Results and Discussion

A. Characteristics of material

Pulverized beetroot at a ratio of 1: 8 resulted in beetroot pulp with the composition of acetic acid 0.06%, total solids 10.22%, gallic acid (calculated as total polyphenol) 0.45%, total sugars 65.02 mg/mL, inhibiting ability 85.59%, and pH 6.38. For 0, 12, and 30 days, the fermentation process increased acetic acid content but decreased total solids, total sugars, and pH, while total polyphenol and inhibiting ability fluctuated, as shown in Table 1. Fig. 3 showed the physical characteristics of the pulp of beetroot and fermented beetroot at 0, 12, and 30 days.

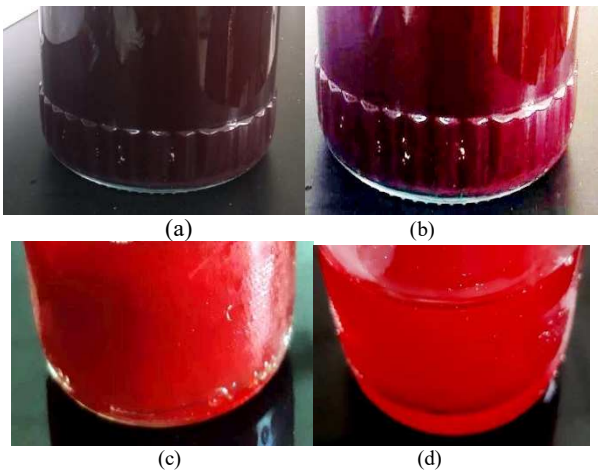


Fig. 3 (a) pulp of beetroot, (b) fermented beetroot (0 day), (c) fermented beetroot (12 days), and (d) fermented beetroot (30 days).

TABLE I
Composition of pulp and fermented beetroot

Components	Type of materials			
	Pulp of beetroot	Fermented beetroot 0 day	Fermented beetroot 12 days	Fermented beetroot 30 days
Acetic acid (%)	0.06	0.16	0. 50	1.10
Total solids (%)	10.22	10.18	10.07	3.50
pH	6.38	4.32	3.64	3.14
Total polyphenol (%)	0.45	0.43	0.61	0.46
Total sugars (mg/mL)	65.02	88.12	65.72	43.32
Inhibition (%)	85.59	64.32	91.74	63.01

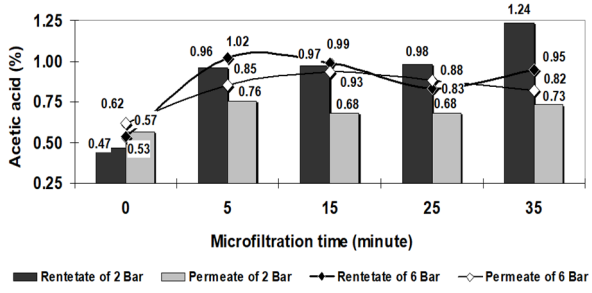
The difference in composition of pulp and fermented beetroot was probably caused by activity of Kombucha culture dominated by *Acetobacter* sp. During fermentation, microorganisms in Kombucha culture using sucrose as carbohydrate source and converted into organic acids, particularly acetic acid in its metabolism process[25]. Thus, it decreased total sugars, total solids, and pH but increased acetic acid. However, the graph was fluctuated and decreased on total polyphenol due to the occurrence of autolysis, in which organic acids would degrade into polyphenol. This change was confirmed as antioxidant ability decreased as fermentation time increased. During fermentation process, not only chemical composition but also physical attributes was shifted, in which color of beetroot biomass became attenuated and clear. Furthermore, it had fresh aroma with

predominant acidic taste due to organic acids formation and pH reduction.

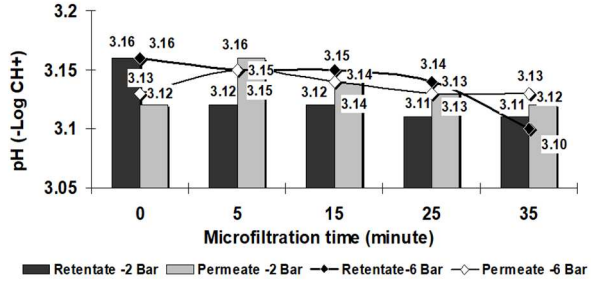
B. Influence of microfiltration process on beetroot biomass

1) Acetic acid (%) and pH: Acetic acid is one of the major organic acids produced by Kombucha culture, dominated by *Acetobacter sp*. bacteria [26]. Acetic acid is analyzed according to the titration method [27]. The MF process indicated succeeded separation in both TMP 6 and 2 bar, as shown by more particles retained on the membrane surface (retentate) in comparison to permeate, as shown in Fig. 4a. This is probably caused by fouling, a condition of the accumulation of all components forming 'cake' and second layer [28], although particle size of acetic acid is smaller (0.001 – 0.01 µm) in comparison with pores size of MF membrane (0.15 µm). At TMP 2 bar, acetic acid increased in retentate until 35 minutes (1.24%). However, at TMP 6 bar, optimum acetic acid was achieved at 5 minutes (1.02%) and decreased until 35 minutes. Higher driven force (6 bar) would compress the components. Thus, it required a shorter time. Meanwhile, at lower driven force (2 bar), component particles accumulate slowly. Thus, it required a longer time. The optimum acetic acid was achieved at TMP 2 bar for 35 minutes with a concentration in retentate (1.24%) and permeate (0.73%). CF-MF system was able to retain acetic acid in retentate 263.83% (2.63-folds) and pass it in permeate 128.07% (1.28-folds) in comparison with the initial process (0 minutes) of 0.47% and 0.57%, or MF membrane separated acetic acid in retentate 112.73% (1.12-folds) and permeate 66.36% in comparison without MF process (1.1%).

pH value indicates the acidity level from biomass before and after the separation process, which is affected by overall organic compounds produced during fermentation (such as acetic acid, malic acid, lactic acid, etc.) and imparted fresh taste like fruits. CF-MF system decreased the pH of retentate and permeated in both TMP as the processing time increased.



(a)

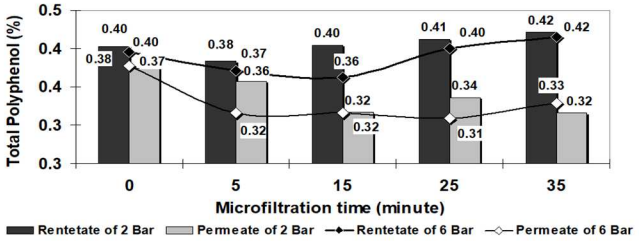


(b)

Fig. 4 Relationship of TMP and time on (a) acetic acid and (b) pH in fermented beetroot by CF-MF.

The level of acidity in the retentate and permeate at TMP 2 bar showed a higher value than TMP 6 bar, as illustrated in Fig. 4b. This was caused by higher TMP (6 bar) as the driving force passes more organic acids; thus, it declined to permeate pH. The optimum pH was obtained at TMP 6 bar for 35 minutes and resulted in retentate with pH 3.13 and permeate with pH 3.10. CF-MF system was able to retain the level of acidity in retentate to 99.05% while allowing it to pass through the membrane as permeate with a level of acidity of 98.1% compared to the initial process (0 minutes) (3.16).

2) Total polyphenol (%), Total Sugars (mg/mL), and Total Solids (%): CF-MF system well-separated gallic acid (calculated as total polyphenol), as more particles were retained on membrane surface than in permeate for all treatment variables, as shown in Fig. 5a. Total polyphenols are determined according to Denise-Folin Method [28]. At TMP 2 bar, total polyphenols were higher in retentate than at TMP 6 bar for all process times. Fouling is probably the main cause of this condition, even though polyphenol has a smaller particle size (0.001 – 0.1 µm) than the pores size of MF membrane (0.15 µm) [23]. On the other hand, higher TMP (6 bar) had total polyphenol passed through in permeate (0.33%) than TMP 2 bar (0.32%), although both TMPs can retain similar total polyphenol in retentate (0.42%). Based on process efficiency, the optimum total polyphenols were achieved at TMP 6 bar for 35 minutes with retentate and permeate, 0.42% and 0.33%, respectively. CF-MF system retained total polyphenol in retentate 105% (1.05-fold) and in permeate 87.16% than the initial process (0 minutes), in which retentate and permeate were 0.40% and 0.38%, respectively.



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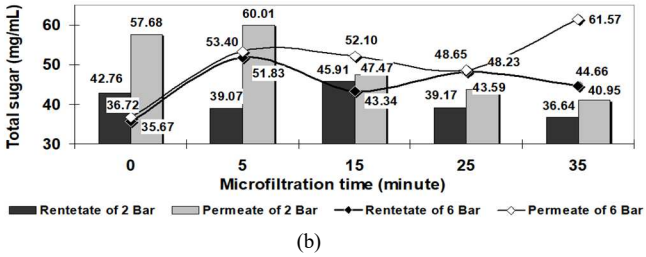

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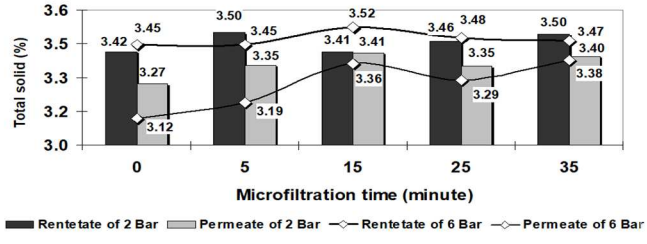


(c)

Fig. 5 Relationship of TMP and time on (a) total polyphenols, (b) total sugars, and (c) total solids in fermented beetroot by CF-MF.

Different trends were shown in total sugars, in which more total sugars passed in permeate than retained on the membrane surface during the filtration process, despite the particle size of sugars and its derivative being smaller (0.001 – 0.1 µm) than the pores size of the membrane (0.15 µm), as shown in Fig. 5b. Total sugars are overall sugars and their derivates contained in biomass and analyzed according to the Phenol Sulphate method [24]. Kombucha culture uses sucrose as a carbon source in its metabolism. CF-MF system showed that higher TMP as driving force (6 bar) caused higher polarization of total sugars on membrane surface at a shorter time (5 minutes) in comparison with lower TMP (2 bar) for 15 minutes.

Furthermore, the CF-MF system can separate total solids, in which more total solids were retained on the membrane surface as retentate than in permeate during the process. Total solids are overall components presented in biomass, both soluble and insoluble due to beetroot fermentation, and determined according to the Gravimetric method [29]. Accumulation on overall components during process formed aggregate with larger particle size than pores size of MF membrane (0.15 um) due to effect of membrane system (including nature and concentration of the feed, the type, and material of membrane, the pore size distribution, operation conditions, such as transmembrane pressure, temperature, flow rate or velocity, turbulence) [30]. Process time was longer achieved at TMP 2 bar due to polarization of total solids being higher on retentate until the final process (35 minutes). In comparison, higher driving force with TMP 6 bar required only 15 minutes to reach an optimum condition in retentate (3.52%) and passed freely in permeate 3.36%, as presented in Fig. 5c. Therefore, CF-MF system was able to retain total solids in retentate 102.03% (1.02-folds) and pass in permeate 105.33% (1.05-folds) than initial process (0 minutes), in which retentate and permeate were 3.45% and 3.12%, respectively.

C. Optimum process condition

The optimum separation process of acetic acid in fermented beetroot was attained at TMP 2 bar for 35 minutes and room temperature, with flow rate ~7.5 L/min. At this condition, retentate had acetic acid 1.24%, gallic acid 0.42%, total solids 3.49%, total sugars 36.64 mg/mL, and pH 3.11, whereas permeate had acetic acid 0.73%, gallic acid 0.31%, total solids 3.39%, total sugars 40.95 mg/mL, and pH 3.12, respectively.

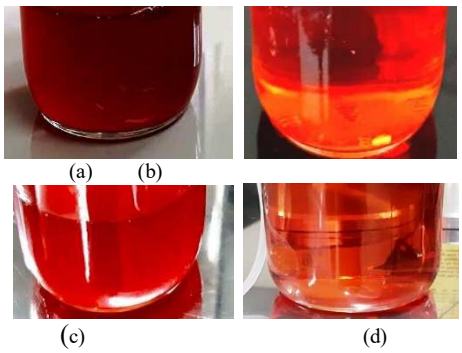


Fig. 6 (a) Retentate and (b) permeate of fermented beetroot at TMP 2 bar, (c) retentate and (d) permeate of fermented beetroot at TMP 6 bar passed through CF-MF at room temperature and flow rate ~7.5 L/min. for 35 minutes.

Hence, CF-MF system was able to retain acetic acid in retentate 263.83% (2.63-folds) and pass it in permeate 128.07% (1.28-folds) than the initial process (0 minute), in which retentate and permeate were 0.47% and 0.57%, respectively. The optimum separation process of gallic acid calculated as total polyphenol in fermented beetroot was obtained at TMP 6 bar for 35 minutes and room temperature with flow rate ~7.5 L/min. At this condition, retentate had gallic acid 0.41%, acetic acid 0.95%, total solids 3.47%, total sugars 44.66 mg/mL, and pH 3.1. Meanwhile, permeate contained gallic acid 0.33%, acetic acid 0.82%, total solids 3.38%, total sugars 61.56 mg/mL, and pH 3.13. Hence, the CF-MF system was able to retain gallic acid in retentate 120.59% (1.21-folds) and pass it in permeate 86.84% compared to the initial process (0 minute), in which retentate and permeate were 0.41% and 0.33%, respectively. Fig. 6a and 6b illustrate retentate (concentrate) and permeate of fermented beetroot passed through CF-MF system at TMP 2 bar for 35 minutes, whereas Fig. 6c and 6d illustrate retentate (concentrate) and permeate of fermented beetroot passed through CF-MF system at TMP 6 bar for 35 minutes.

D. Antioxidant activity (inhibition)

Antioxidant activity (inhibition) was conducted on retentate and permeated with the highest acetic acid and gallic acid content, at optimum process conditions, in which driven forces were TMP 2 bar and 6 bar for 35 minutes respectively. Inhibition of retentate at lower TMP (2 bar) were higher (62.45%) than retentate at TMP 6 bar (56.48%). A different trend was shown in the inhibition of permeate at both TMPs, in which TMP 6 bars has higher inhibition (52.43%) than permeate at TMP 2 bar (47.62%). This indicated that CF-MF system provides better inhibition in retentate at TMP 2 bar and in permeate at TMP 6 bar. The difference in inhibition activity is probably caused by retentate optimization at TMP 2, and 6 bar achieved based on acetic acid (1.24%) and gallic acid (0.41%). In this condition, permeate had lower acetic acid content (0.73%) and gallic acid (0.33%) than retentate, resulting in low inhibition of permeate, 52.43% and 47.62%, respectively. This showed the relationship of organic acids and antioxidant ability. At both TMP 2 and 6 bar, retentate had stronger inhibition than permeate, as shown in Fig. 7. Overall, CF-MF system at TMP 2 bar for 35 minutes increased retentate inhibition (25.12%) but decreased permeate inhibition (4.59%) in comparison with the initial process or feed (49.91%). At TMP 6 bar for 35 minutes, the CF-MF system gained retentate and permeate inhibition by 11.25% and 3.27%, respectively, compared with the initial process or feed (0 minutes).

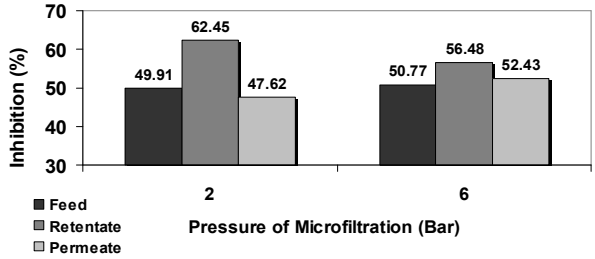
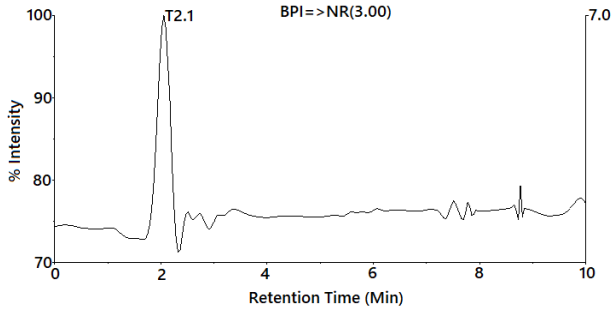


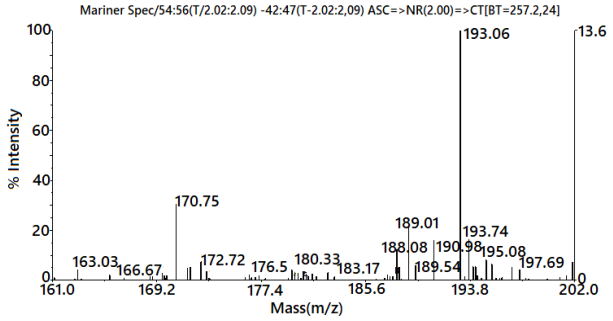
Fig. 7 Relationship of type of fermented beetroot and TMP on inhibition ability via CF-MF at flow rate ~7.5 L/min. for 35 minutes.

E. Influence of CF-MF process on gallic acid and acetic acid monomers

1) Identification of gallic acid and acetic acid standard: Identification of gallic acid and acetic acid were performed by LC-MS. Gallic acid and acetic acid have a molecular weight (MW) of 91 Dalton (Da) and 61 Da. LC-MS method has been used to identify a compound based on MW difference, in which the possibility is as M^+, M^+ Na^+, $2M^{++}$ or $2M^+$, Na^+.



(a)



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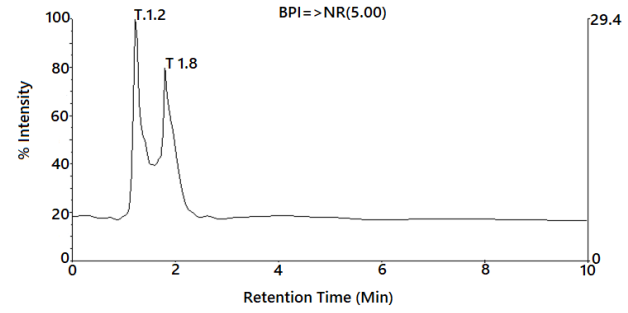

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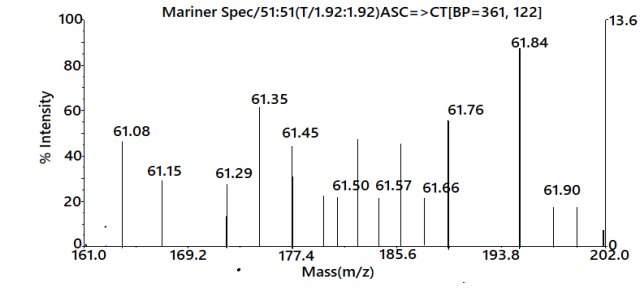


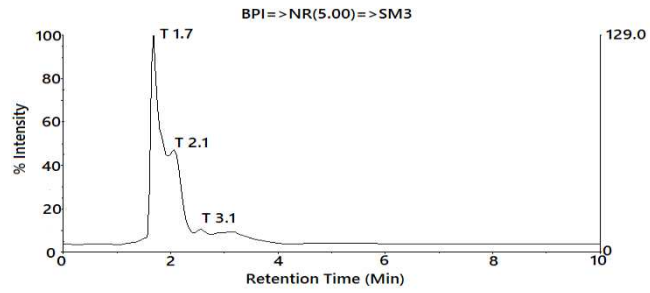
(d)

Fig. 8 (a) Chromatogram of standard gallic acid and (b) mass spectra of standard gallic acid (M+Na+), (c) chromatogram of standard acetic acid and (d) mass spectra of standard acetic acid (M+).

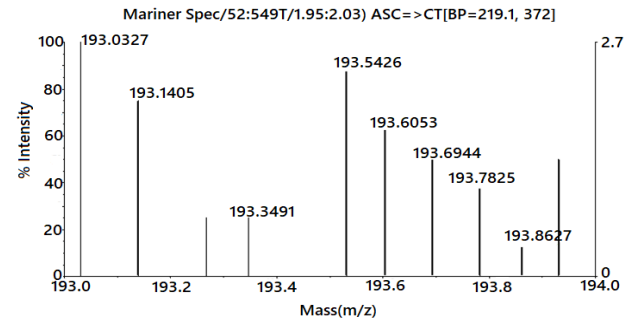
The operation conditions of LC-MS were injection volume 5 µL, flow rate 0.2 mL/min., methanol as eluent, and using a C-8 column (15 mm x 2 mm). Chromatogram of gallic acid standard showed one (1) peak (T1.3) with a retention time of 0 – 10 minutes at relative intensity 100%. Mass spectra at T1.3 between *m/z* 161.0 and 202.0 indicated gallic acid monomer, which is predominated by gallic acid monomer with MW 193.06 Da. ($M+Na^+$), as illustrated in Fig. 8a and 8b. Chromatogram of acetic acid standard exhibited 2 peaks, which is dominated at T1.2 with retention time 0 – 10 minutes and relative intensity 100%. Mass spectra at T1.2 over the *m/z* range of 61.0 – 62.0 indicated an acetic acid monomer with MW 61.84 Da. (M+) with relative intensity 100%, as illustrated in Fig. 8c and 8d.

2) Identification of gallic acid and acetic acid monomers: Gallic acid and acetic acid were identified in the optimum condition for both components at TMP 6 and 2 bar for 35 minutes, respectively. Chromatogram of permeate from fermented beetroot at optimum condition (room temperature, flow rate ~7.5 L/min. and TMP 6 bar for 35 minutes) displayed peak domination at T1.7 with retention time 0 – 10 minutes, as shown in Fig. 9a.

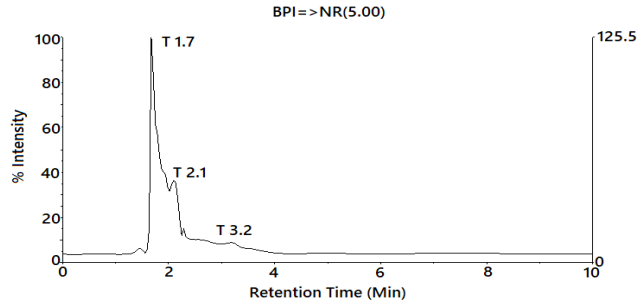
Mass spectra at T1.7 presented nine gallic acid monomers between *m/z* 193.03 and 193.90, predominantly gallic acid with MW 193.0327 Da ($M+Na^+$), as shown in Fig. 9b. Chromatogram of permeate from fermented beetroot at optimum treatment (room temperature, flow rate ~7.5 L/min. and TMP 2 bar for 35 minutes) indicated peak domination T1.7 with retention time 0 – 10 minutes, as shown in Fig. 9c. Mass spectra at T1.7 exhibited sixteen acetic acid monomers between *m/z* 61.0077 and 61.9688, predominantly acetic acid monomer with MW 61.1652 Da ($M+Na^+$), as illustrated in Fig. 9d.



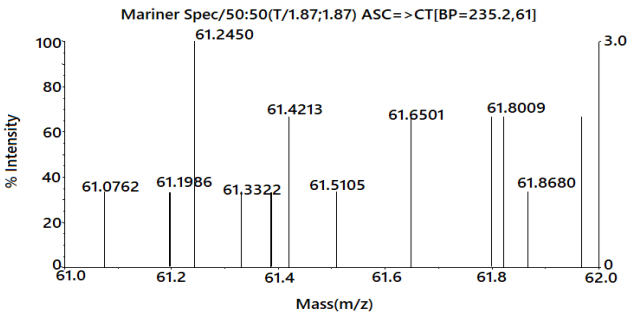
(a)



(b)



(c)



(d)

Fig. 9 (a) Chromatogram of gallic acid and (b) mass spectra of gallic acid at room temperature, flow rate ~7.5 L/min. and TMP 6 bar for 35 minutes, (c) chromatogram of acetic acid and (d) mass spectra of acetic acid at room temperature, flow rate ~7.5 L/min. and TMP 2 bar for 35 minutes

IV. Conclusion

The CF-MF system has the potential to separate total polyphenol and acetic acid. The longer separation process increased total polyphenol, acetic acid, and total solids but decreased pH value. However, total sugars fluctuated in retentate for both TMPs. Based on acetic acid and total polyphenol concentrations, the optimum process was achieved at TMP 2 and 6 bar for 35 minutes with a flow rate ~7.5 L/min and room temperature. At optimum conditions, CF-MF was able to retain acetic acid and gallic acid in each retentate by 263.83% (2.63-folds) and 120.59% (1.21-folds), which increased inhibiting ability by 25.12% and 11.25%, respectively. Additionally, acetic acid was retained in permeate by 128.07% (1.28-folds) but decreased inhibiting ability by 4.59% and 86.84%, respectively. Permeate at TMPs 2 bar and 6 bar for 35 minutes is dominated by monomers of acetic acids and gallic acids with MWs 61.2450 Da (M^+) and 193.0327 Da (M^+) and relative intensity of 100 %, respectively.

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