

## Investigating the influence of pectin content and structure on its functionality in bio-flocculant extracted from okra

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### ABSTRACT

Okra extract is known to have potential application as a bio-flocculant for wastewater treatment. However, no research to date has given insight into the components responsible for the flocculating ability of okra extract or its flocculating mechanism. The work presented here addresses this knowledge gap showing that pectin, especially pectin homogalacturonan (HGA) regions, appear to be the polysaccharides responsible for the flocculating ability of okra extract. The way pectin works in flocculation may be best explained by a polymer bridging mechanism. Specifically, a linear relationship between okra bio-flocculating ability and pectin homogalacturonan region to rhamnogalacturonan-I region weight ratio (HGA/RG-I) was found ( $y = 2.0x + 47.6$ ,  $R^2 = 0.93$ , when GalA content > 300 mg/g extract), which was also validated using commercial citrus peel pectin.

### 1. Introduction

Flocculation is one of the most widely used solid-liquid separation processes for the removal of insoluble suspended solids and colloids and/or soluble organic matter in water treatment (Renault et al., 2009). This industrial process, with the help of flocculant, combines those fine divided or dispersed particles together into aggregates, then facilitates their removal in subsequent sedimentation, floatation and filtration stages and thus causes clarification of the system (Renault et al., 2009; Sharma et al., 2006).

Although some inorganic mineral additives and synthetic organic polymers are already widely applied as flocculants, with increasing awareness of the harm caused by chemical flocculants and implementation of more stringent environmental regulations, bio-flocculants have attracted the interest due to advantages such as biodegradability, nontoxicity and availability from reproducible agricultural resources (Lee et al., 2014a, Lee et al., 2014b). Many carbohydrate polymers have been reported as effective flocculation agents, for example starch-based polymers (Pal et al., 2005; Wei et al., 2008) and chitosan (Zeng et al., 2008). Bio-flocculants are often used in drinking water treatment and food-related industries, where chemical flocculants can't be used (Bolto & Gregory, 2007; Lu et al., 2014); for

example, for the removal of colloids, suspended particles, and coloring matters from sugarcane juice in syrup production (Laksameethanasana et al., 2012). There are also many plant-based bio-flocculants including extracts from okra (*Abelmoschus Esculentus*) (Lee et al., 2015), mallow (*Malva sylvestris*) (Anastasakis et al., 2009), and modified konjac polysaccharides (Xie et al., 2007) etc.

Okra has attracted considerable attention as a bio-flocculant source. Okra is an inexpensive and readily available plant in tropical countries all year round (Lee et al., 2015). Its extract has been shown to be an effective bio-flocculant and is efficient in removing turbidity in lower dosage than other plant-based bio-flocculants (Anastasakis et al., 2009). Okra extracts can be obtained by microwave-assisted extraction (MAE), which has been continuously reported as an alternative extraction method to conventional-solvent extraction (CSE) as it can enhance and/or accelerate the extraction of bio-active compounds (Bagherian et al., 2011; Fishman et al., 2006). Lee (2017) have shown that the use of microwave-assisted extraction to obtain okra flocculant extract means that lower dosages can be used to treat kaolin clay solution and give the same effects as extracts obtained through different methods such as CSE, reducing the suspended solids of a kaolin clay solution by greater than 99.5 %.

The mechanisms of okra flocculation ability are believed to be

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through the combined effects of charge neutralisation and polymer bridging. Charge neutralisation is the major mechanism when the flocculant and the adsorption site are of opposite charge. In this case the flocculation occurs as the result of reduced surface charge of the particles and hence the decreased electrical repulsion force between colloidal particles, which allows the formation of van der Waals force of attraction to encourage initial aggregation of colloidal and fine suspended materials to form microfloc (Lee et al., 2014a, Lee et al., 2014b). However, if too much polymer is used, a charge reversal can occur and the particles will again become dispersed, but with a positive charge rather than a negative charge (Lee et al., 2014b). By contrast, polymer bridging occurs when long chain polymers with high molecular weight and low charge density adsorb on particles in a way that long loops and tails extend or stretch into solution, which creates the possible interaction and so-called ‘bridging’ of these polymers with particles (Biggs et al., 2000). However, the amount of polymers absorbed should not be excessive, otherwise the particle surface would be overly coated with polymers such that no sites are available to ‘bridge’ with other particles, and this is called restabilisation (Sher et al., 2013).

Okra bio-flocculants are mainly extracted from okra pulp (ca. 72 g/100 g of fresh okra) using water as the solvent and they comprise a highly viscous solution with a slimy appearance originating from okra’s high polysaccharide content (Sengkhampan et al., 2009). The okra polysaccharide contents were found rich in L-galacturonic acid, L-rhamnose, D-galactose and D-glucose (Deters et al., 2005; Lengsfeld et al., 2004). Tomoda, Shimada, Saito, and Sugi (1980) suggested the main chain of okra mucilage has the repeating structure 1,2- $\alpha$ -L-rhamnopyranose-1,4- $\alpha$ -D-galacturonyluronic acid. These findings indicated that the main components in the okra mucilage may come from pectin. Pectins are complex heteropolysaccharides found in the primary cell wall and middle lamella of terrestrial plants consisting of two major structural domains: a). homogalacturonan (HGA), a linear homopolymer of  $\alpha$ -1,4-linked-D-galacturonic acid (GalA), which can be methylsterified and acetylated to various degrees (Harding et al., 2017) and is regarded as “smooth” pectin; b). rhamnogalacturonan-I (RG-I), repeating units of the disaccharide 1,2- $\alpha$ -L-rhamnose-1,4- $\alpha$ -D-galacturonic acid (same structure in the okra mucilage as suggested above in Tomoda et al. (1980)) with many of the rhamnose residues connecting to side chains of linear and branched  $\alpha$ -L-arabinofuranosyl (Araf), and/or  $\beta$ -D-galactopyranosyl (Galp) residues. Due to its highly branched nature, RG-I is often referred to as “hairy” pectin (Willats et al., 2001). Tomoda et al. (1980) has proved that okra extracts contain pectin RG-I region, and Lengsfeld et al. (2004) have suggested that there is more pectin HGA region than RG-I region. Furthermore, some studies have proven that pectins have good flocculating abilities and may be applied as flocculating agent, for example in the work of Yokoi et al. (2002) using apple pectin.

Although many researchers have studied okra bio-flocculants in terms of their flocculating abilities, no research to date has characterised the carbohydrate polymers present in the extracts and correlated this information with their behaviour as flocculating agents. The aim of this study was to investigate whether the flocculating property of a flocculant extracted from okra is due to the pectin that is present, and if it is due to pectin, which regions of pectin. The okra flocculants extracted were isolated using iso-propanol (IPA) into alcohol-insoluble fraction (AIF) and the alcohol-soluble fraction (ASF), where AIF would be mainly pectin while ASF would be mainly hemicellulose and non-structural cell wall components like protein and ash. Different regions of pectin were extracted using deionised water, acid and alkali, where acid would be beneficial to the extraction of HGA region pectin while destroying RG-I side chains, alkali would be beneficial to RG-I extraction while destroying HGA, and water can help to preserve both regions (Koubala et al., 2008; Mao et al., 2019). Therefore, the detailed objectives of this study are to:

- 1 Compare the yield and chemical compositions of okra flocculants,

AIF and ASF using deionised water, acid and alkali as extraction solvents.

- 2 Compare the effectiveness of the okra flocculants, AIF and ASF in reducing the suspended solids in a kaolin clay solution.
- 3 Relate okra extract composition to its bio-flocculating properties.

It is hoped that in gaining understanding of the components responsible for okra’s flocculating properties, better understanding of how to design and optimise industrial extraction processes to maximise flocculating properties, and also how to identify other natural feed-stocks with flocculating properties, will be achieved.

## 2. Materials and methods

### 2.1. Materials

The okra was bought fresh from a local shop (Nottingham, UK) and used within a few days of purchase. The nylon filter cloth (35-mesh size) was used for filtration in the sludge dewatering. The kaolin powder (Polwhite B China Clay, chemical formula  $\text{Al}_2\text{Si}_2\text{O}_5(\text{OH})_4$ ) was purchased from Richard Baker Harrison Ltd. (Manchester, UK). Hydrogen chloride (37 % w/w HCl), sodium hydroxide (50 % w/w NaOH), iso-propanol (IPA) and commercial citrus peel pectin (GalA content  $\geq 74.0$  % w/w and methoxy groups  $\geq 6.7$  % w/w of dry basis) were purchased from Sigma Aldrich (Dorset, UK).

### 2.2. Sample preparation

All okras were washed with deionised water to remove any contaminants. The upper crown head and the seeds inside the pods were removed and the pods were hand sliced into cubes of 5–10 mm, which was found as the optimal size for okra flocculants extraction (Lee, 2017). The moisture content of the okra samples was obtained by oven drying at 60 °C until a constant weight was obtained, which was  $(88.42 \pm 0.34)$  %.

For bio-flocculating ability evaluation using a Jar Test (Rice et al., 2012), 80 g/L kaolin solutions were prepared by slowly adding 80 g of powder into 1 L of water, whilst stirring at 700 rpm for 20 min to ensure homogeneity. The pH of this slurry was adjusted to within the range of  $4.82 \pm 0.26$  and the suspended solids (SS) of the slurry were measured as  $(80 \pm 0.78)$  g/L.

### 2.3. Bio-flocculants and pectin extraction

10 g sliced okra pod was added into 35 mL extracting solvent with solid to liquid ratio (S/L) of 1:3.5 which was found as the optimal S/L for okra flocculants extraction (Lee, 2017). Solvents used were deionised water, hydrochloric acid at pH 2 and sodium hydroxide solution at pH 12. The extraction was microwave-assisted and carried out using a Miniflow 200SS (Sairem, France) single mode microwave heating system at 2.45 GHz. A temperature probe was inserted into the reaction vessel. A maximum incident power of 200 W was applied to the system to achieve the temperature set-point. The reflected power was adjusted to zero before the treatment, so that the absorbed power approached the incident power. The treatment temperature was chosen at temperature of 90 °C as this was the temperature suggested as the optimum treatment temperature by Lee (2017). The treatment times were 5, 10 and 15 min. and the optimum treatment time would be determined by extract yields. After heating, solid okra residue was removed by centrifugation at 3900 rpm for 30 min. The resulting supernatant was okra flocculant, which was oven dried for 24 h at 60 °C to determine yield.

To obtain pectin, an equal volume of IPA was added to the extracted okra flocculant and samples were stored overnight at 4 °C for pectin precipitation. The samples were then centrifuged for another 40 min at 3900 rpm. This supernatant was called alcohol-soluble fraction (ASF), which was mainly hemicellulose and other plant cell interior and wall

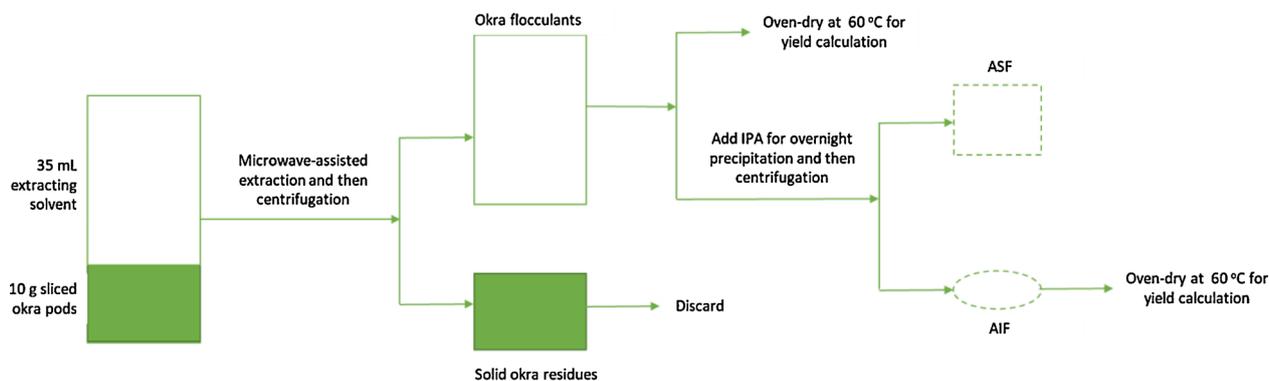


Fig. 1. Flow diagram for okra flocculants and pectin extraction.

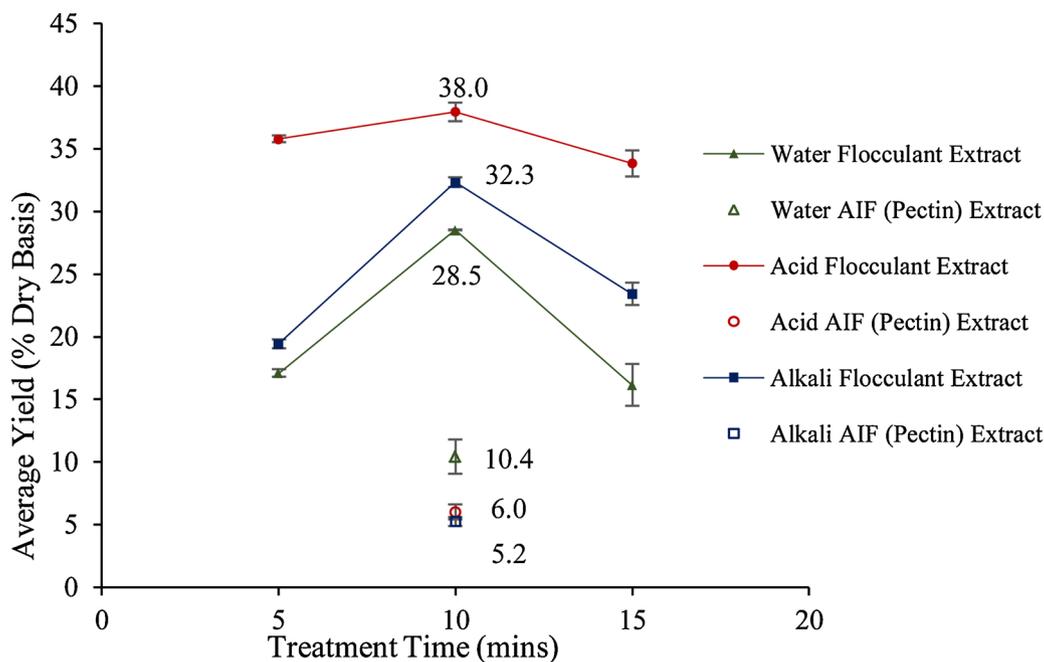


Fig. 2. Yields of okra flocculants and AIF extracted using water, acid and alkali.

components. The precipitate was called alcohol-insoluble fraction (AIF), which was mainly pectin. AIF was oven dried for 24 h at 60 °C to determine yield. Fig. 1 shows a schematic of the extraction method.

2.4. Yield calculation

The yield of okra flocculant was calculated by:

$$\% \text{ yield of okra flocculant} = \frac{\text{Dry weight of okra flocculants}}{\text{Dry weight of okra added}} \times 100\% \quad (1)$$

The yield of okra AIF was calculated by:

$$\% \text{ yields of okra AIF} = \frac{\text{Dry weight of okra AIF}}{\text{Dry weight of okra added}} \times 100\% \quad (2)$$

2.5. Jar Test procedures and suspended solid (SS) measurements

The flocculating abilities of the okra flocculants, okra ASF and AIF extracts were determined using the Jar Test with a commercially available Jar Tester (ET 720, Lovibond, Germany). All measurements of SS in this study were conducted according to the Standard APHA Method 2540D (Rice et al., 2012). Samples were oven-dried at a mild temperature 40 °C. Water was then added to the dry extracts to make

stock solutions with a concentration of 25 g/L, stirring at 800 rpm until all the extracts were dissolved and distributed homogeneously. 1 mL stock solution was injected into 100 mL (0.1 L) of homogeneous kaolin clay solution whilst stirring at 700 rpm for 2 min. and then 350 rpm for 2 min. After 30 min. settling, the solution was filtered using a Buchner funnel and 3 layers of the nylon filter cloth; the resulting filtrate was used to determine the SS remaining in the solution after flocculation and filtration. All measurements of SS in this study were conducted according to the Standard APHA Method 2540D (Rice et al., 2012). After filtration using 47 mm filter papers (Fisher Scientific, UK), the SS retained on the filter paper was oven dried at 60 °C.

The SS after flocculation (g/L) was calculated by:

$$SS_{\text{after flocculation}} = \frac{\text{Dry weight of SS on filter paper (g)}}{0.1 \text{ (L)}} \quad (3)$$

The reduction in SS (%) was calculated by the following equation, where  $SS_{\text{slurry}}$  was 80 g/L mentioned in Section 2.2:

$$\text{The reduction in SS (\%)} = \frac{SS_{\text{slurry}} - SS_{\text{after flocculation}}}{SS_{\text{slurry}}} \times 100\% \quad (4)$$

**Table 1a**

Chemical and physical properties of okra flocculants extracts, AIF extracts and ASF extract using solvent water, acid and alkali at optimum treatment condition. Where, the yields of ASF was calculated based on mass balance and Yields of ASF = Yields of AIF; Yields of AIF; GalA is galacturonic acid; Rha is rhamnose; Ara is arabinose; Gal is galactose; Glu is glucose; Xyl is xylose; Total pectic sugars = GalA + Rha + Ara + Gal + Glu + Xyl; Total RG-I region = Rha + Ara + Gal; HGA/RG-I = GalA/(Rha + Ara + Gal), which represents the ratio of pectin HGA region to pectin RG-I region;  $[\eta]$  represents average intrinsic viscosity. The  $[\eta]$  of ASF are not applicable (N/A) because the viscosity of all three solutions are very close to solvent water.

	Optimum yields g/g dried okra biomass $\times 100\%$	GalA	Rha	Ara	Gal	Glu	Xyl	Total sugar	Total RG-I region	HGA/RG-I	DM	$[\eta]$
		mg/g extract	mg/g extract	mg/g extract	mg/g extract	mg/g extract	mg/g extract	mg/g extract	mg/g extract	Weight ratio	%	ml/g
Okra water flocculant extracts	28.5 $\pm$ 1.0	512.2 $\pm$ 2.0	3.3 $\pm$ 0.6	6.1 $\pm$ 0.7	29.0 $\pm$ 0.2	105.7 $\pm$ 0.5	27.1 $\pm$ 0.9	683.4 $\pm$ 4.9	38.4 $\pm$ 1.5	13.3 $\pm$ 1.5	17.1 $\pm$ 1.7	255 $\pm$ 1
Okra water AIF (Pectin)	10.4 $\pm$ 1.4	806.1 $\pm$ 3.4	5.4 $\pm$ 0.4	12.1 $\pm$ 0.2	18.9 $\pm$ 0.1	108.7 $\pm$ 0.8	37.4 $\pm$ 1.0	988.6 $\pm$ 5.9	36.4 $\pm$ 0.7	22.1 $\pm$ 1.1	15.9 $\pm$ 1.7	1740 $\pm$ 135
Okra water ASF	18.1 $\pm$ 2.4	150.3 $\pm$ 0.8	1.7 $\pm$ 0.1	1.0 $\pm$ 0.3	21.9 $\pm$ 1.1	100.0 $\pm$ 1.3	19.9 $\pm$ 1.2	294.8 $\pm$ 4.8	24.6 $\pm$ 1.5	6.1 $\pm$ 1.3	43.3 $\pm$ 1.6	N/A
Okra acid flocculant extracts	38.0 $\pm$ 0.8	355.9 $\pm$ 1.2	2.8 $\pm$ 0.1	3.3 $\pm$ 0.7	26.8 $\pm$ 0.7	98.5 $\pm$ 1.4	22.5 $\pm$ 1.2	509.8 $\pm$ 11.6	32.9 $\pm$ 7.8	10.8 $\pm$ 1.7	22.8 $\pm$ 1.5	526 $\pm$ 0
Okra acid AIF (Pectin)	6.0 $\pm$ 0.6	712.8 $\pm$ 2.9	3.9 $\pm$ 0.9	9.2 $\pm$ 0.1	14.5 $\pm$ 0.9	87.4 $\pm$ 1.2	37.5 $\pm$ 1.8	865.3 $\pm$ 7.8	27.6 $\pm$ 1.8	25.8 $\pm$ 2.8	6.8 $\pm$ 0.4	1360 $\pm$ 58
Okra acid ASF	32.0 $\pm$ 1.4	130.4 $\pm$ 0.6	1.3 $\pm$ 0.1	0.6 $\pm$ 0.0	24.0 $\pm$ 0.3	88.4 $\pm$ 1.1	19.5 $\pm$ 2.1	264.2 $\pm$ 4.2	25.9 $\pm$ 0.4	5.0 $\pm$ 1.0	77.7 $\pm$ 1.4	N/A
Okra alkali flocculant extracts	32.3 $\pm$ 0.4	302.3 $\pm$ 1.1	3.2 $\pm$ 0.1	3.4 $\pm$ 0.9	27.8 $\pm$ 1.0	101.7 $\pm$ 1.2	22.1 $\pm$ 0.2	460.5 $\pm$ 4.5	34.4 $\pm$ 2.0	8.8 $\pm$ 1.1	25.8 $\pm$ 1.6	725 $\pm$ 1
Okra alkali AIF (Pectin)	5.2 $\pm$ 0.4	608.2 $\pm$ 2.1	4.8 $\pm$ 0.2	11.0 $\pm$ 0.3	17.3 $\pm$ 0.5	109.8 $\pm$ 0.3	36.8 $\pm$ 0.1	787.9 $\pm$ 3.5	33.1 $\pm$ 1.0	18.4 $\pm$ 1.1	20.1 $\pm$ 1.3	4890 $\pm$ 31
Okra alkali ASF	27.1 $\pm$ 0.8	116.9 $\pm$ 0.6	2.5 $\pm$ 0.1	0.8 $\pm$ 0.0	21.5 $\pm$ 1.0	90.7 $\pm$ 0.4	19 $\pm$ 1.2	251.4 $\pm$ 3.3	24.8 $\pm$ 1.1	4.7 $\pm$ 1.7	87.4 $\pm$ 1.8	N/A

## 2.6. Galacturonic acid analysis

The GalA content was determined based on the method developed by Filisetti-Cozzi & Carpita (1991). Soluble pectin extracts, lyophilised using a LyoDry freeze dryer (Mechatech Systems, Bristol, UK), were diluted to give an estimated concentration of 125  $\mu$ g/mL in D.I water. Then the solutions were dispensed into pyrex boiling tubes, followed by the addition of 40  $\mu$ L 4 M potassium sulphamate and 2.5 mL of concentrated sulphuric acid. The mixture was heated to 99  $^{\circ}$ C for 20 min., which allowed the break-down of the HGA backbones into GalA. The solution was then cooled under running water. 80  $\mu$ L of a 0.15 wt% m-hydroxydiphenyl in 0.5 wt% NaOH solution was added into each tube and shaken on an orbital shaker to mix. The tubes were left to stand for 10 min to allow a pink colour to develop. The absorbance of the pink solution was determined by UV-vis chromatography (Jenway model 7315, Cole-Parmer Ltd UK) at a wavelength of 525 nm. Zero absorbance reference was set with deionised water. The GalA standard solution was made at concentrations from 0 ~ 97 mg/L and was treated the same way as above. The results and error bars of GalA composition were determined by 4 repeats ((2 freeze-dried samples)  $\times$  (duplication measurements of each sample in UV-vis), such that the error bars include both the errors from extraction method and sugar analysis method).

## 2.7. Neutral sugar analysis

The freeze-dried pectin extract was hydrolysed with concentrated sulphuric acid using the same method as in the GalA assay. The neutral sugar analysis followed the method in Mao et al. (2019). After the pre-hydrolysis, 100  $\mu$ L of supernatant was added to 10 mL of 10 mM NaOH. 1 mL of the resulting solution was used for sugar analysis. High-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (Dionex, UK) using a CarboPac PA20 column and software Chromeleon was used. 10 mM NaOH (Solution A) was used as eluent and 200 mM NaOH (Solution B) as the mobile phase; retention gradient and time as -10 ~ -5, 0.5 mL/min, 100 % Solution A; -5 ~ 14, 0.4 mL/min, 100 % Solution B; stand-by, 0.1 mL/min. Mixtures of sugar standards (L-rhamnose, L-arabinose, D-galactose, D-glucose and D-xylose, all chemicals from Sigma Aldrich (Dorset, UK)) at various concentrations (1 ~ 20 mg/L) were used as external standards for identification and quantification. The results and error bars of neutral sugar composition were determined by 4 repeats ((2 freeze-dried samples)  $\times$  (duplication measurements of each sample in Dionex), in which way the error bars include both the errors from extraction methods and sugar analysis methods).

## 2.8. Degree of methylation (DM) measurements

The DM of pectin, key parameters for HGA pectin functionality, were estimated as the ratio of the molar percent of methanol to the molar percent of GalA. The determination of DM was based on Müller-Maatsch et al. (2014). Extract was incubated (2 h, room temperature) with 1 mL 0.4 M NaOH in D<sub>2</sub>O and 0.1 mL of internal standard (TSP, 0.2 mg/mL in D<sub>2</sub>O). After centrifugation, the supernatant was then transferred into a NMR tube. Spectra were collected using <sup>1</sup>H NMR at 298 K, with 32 K complex points, using a 90 $^{\circ}$  pulse length. Sixteen scans were acquired with a spectral width of 7196.8 Hz, an acquisition time of 2.53, and a relaxation delay (d1) of 5 s. The quantitative determination of methanol was obtained by manual integration of the corresponding signals at 3.358 ppm and comparison with the TSP area. Through the content of methanol and the content of galacturonic acid, the degree of methylation was calculated, using the following equation:

$$DM(\%) = \frac{\text{mol of methanol}}{\text{mol of galacturonic acid}} \times 100\% \quad (5)$$

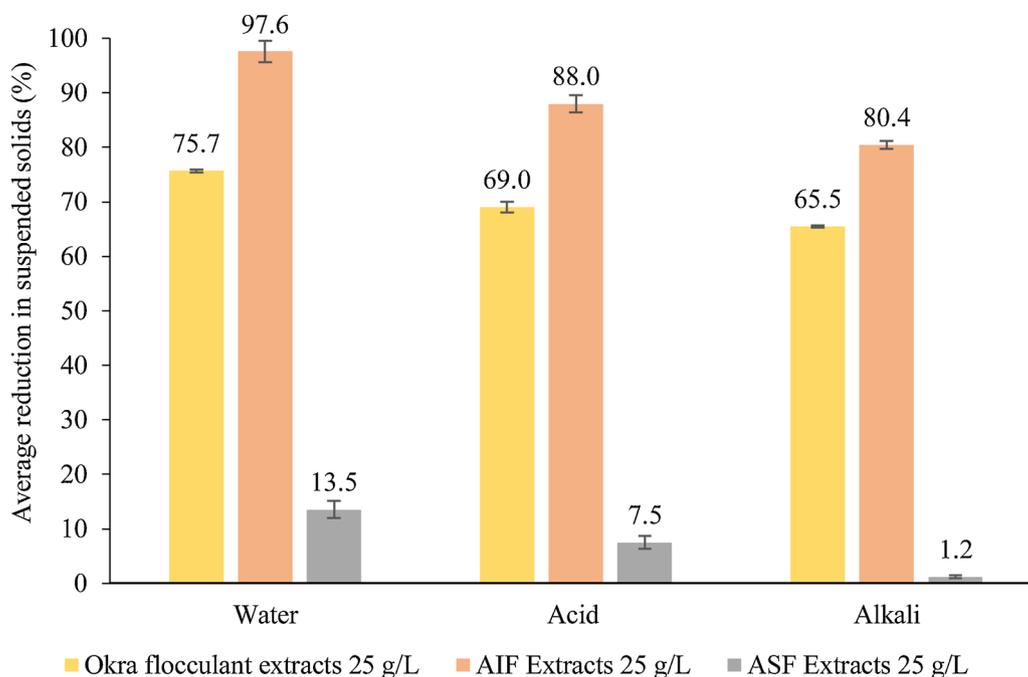


Fig. 3. Flocculation abilities of okra flocculant extracts, AIF extracts and ASF extracts.

### 2.9. Intrinsic viscosity ( $[\eta]$ ) measurements

A pH 7 buffer was made of 4.595 g/L sodium phosphate dibasic dodecahydrate ( $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ ), 1.561 g/L potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ) and 2.923 g/L sodium chloride ( $\text{NaCl}$ ) (all chemicals were purchased from Sigma Aldrich (Dorset, UK)). The freeze-dried okra extracts were dissolved in this buffer and diluted to 5 concentrations of 0.1, 0.075, 0.05, 0.025 and 0.01 mg/mL. 2 mL of solution was injected into a U-tube capillary viscometer (Camlab, UK). The viscometer tube was placed into the water chamber, and the water temperature was set and controlled to 25.0 °C in advance. The time taken for the level of the solution to flow down through two marks on the tube was measured and recorded automatically. For each concentration of the extract, duplication runs were taken; and for each run, the time was measured after temperature equilibration three times (in total there were 6 data points for one concentration of each extract solution). The intrinsic viscosity was calculated three times based on the Huggins, Kraemer and Solomon-Ciuta equations (Harding, 1997). The results of intrinsic viscosity were shown as the average intrinsic viscosity  $[\eta]$  calculated from the three equations:

$$\text{Huggins equation: } \frac{\eta_{sp}}{c} = [\eta] + k_H [\eta]^2 c \quad (6)$$

$$\text{Kraemer equation: } \frac{\ln \eta_r}{c} = [\eta] - k_K [\eta]^2 c \quad (6)$$

$$\begin{aligned} \text{Solomon - Ciuta equation: } [\eta] &= \frac{[2(\eta_{sp} - \ln \eta_r)]^{1/2}}{c} \\ &= \frac{[2(\eta_r - 1 - \ln \eta_r)]^{1/2}}{c} \end{aligned} \quad (6)$$

Where,  $\eta_r$  is the relative viscosity shown as the ratio of the increment in the solution viscosity  $\eta$  to that of the pure solvent  $\eta_0$ , ( $\eta_r = \eta/\eta_0$ );  $\eta_{sp}$  is the specific viscosity of a solution of concentration  $c$ , and it is defined as  $\eta_{sp} = \left(\eta - \frac{\eta_0}{\eta_0}\right) = \eta_r - 1$ ;  $k_H$  and  $k_K$  are the (dimensionless) Huggins and Kraemer constants respectively.

## 3. Results and discussion

### 3.1. Optimisation of okra flocculants and AIF extraction yields

The yields of okra flocculants extracted using water, hydrochloric acid at pH 2 and sodium hydroxide solution at pH 12 at treatment time 5–15 min. and 90 °C were calculated based on Eq. (1). The yields of AIF were found based on the optimum extraction condition of flocculants and calculated using Eq. (2). Results are shown in Fig. 2. The optimum treatment time of flocculants using all three solvents was 10 min.. These results were similar to the work of (Lee, 2017), where the optimum treatment time was also 10 min. using MAE and 70 °C. After 10 min., the yields started to decrease, which is mostly likely due to the extracts' degradation (Bagherian et al., 2011). Acid flocculants had the highest optimum yields at 38.0 %, followed by alkali flocculants at 32.3 % and water flocculants at 28.5 %. This is thought to be because compared to neutral pH solvent, acidic and alkaline conditions help to open the cell wall structure, which leads to better solubility of cell wall components into the extracting solvents and thus higher yields (Adetunji et al., 2017; Yeoh et al., 2008).

At 10 min., the best AIF yield of 10.4 % came from water AIF, followed by 6.0 % for acid AIF and 5.2 % for alkali AIF. Additionally, Table 1a shows the yields of ASF calculated based on mass balance, assuming there was no extract loss during handling in yield measurements. Converse to AIF yields, water ASF had the worst ASF yields of 18.1 %, while alkali and acid extract ASF yields were 27.1 % and 32.0 % respectively. This is because although acid and alkaline conditions help to open the cell wall structure, they affect not only pectin but also other cell wall components (for example, cellulose and hemicellulose), which means the high flocculant yields from acid and alkali extractions are not necessarily due to increased AIF (pectin) extraction but other cell components, and this explains the fact that high flocculant yield under acidic and alkaline conditions doesn't correlate with high AIF yields. The likely reason for the lower AIF yields from acid and alkali extracts compared with water extracts is that extremes in pH degrade the pectin, and this will be discussed further with the extract chemical compositions presented in Section 3.2. Previous studies have also shown promising yields of okra extracts (Anastasakis et al., 2009; Lee et al., 2015) but they only used water as the solvent. Although this

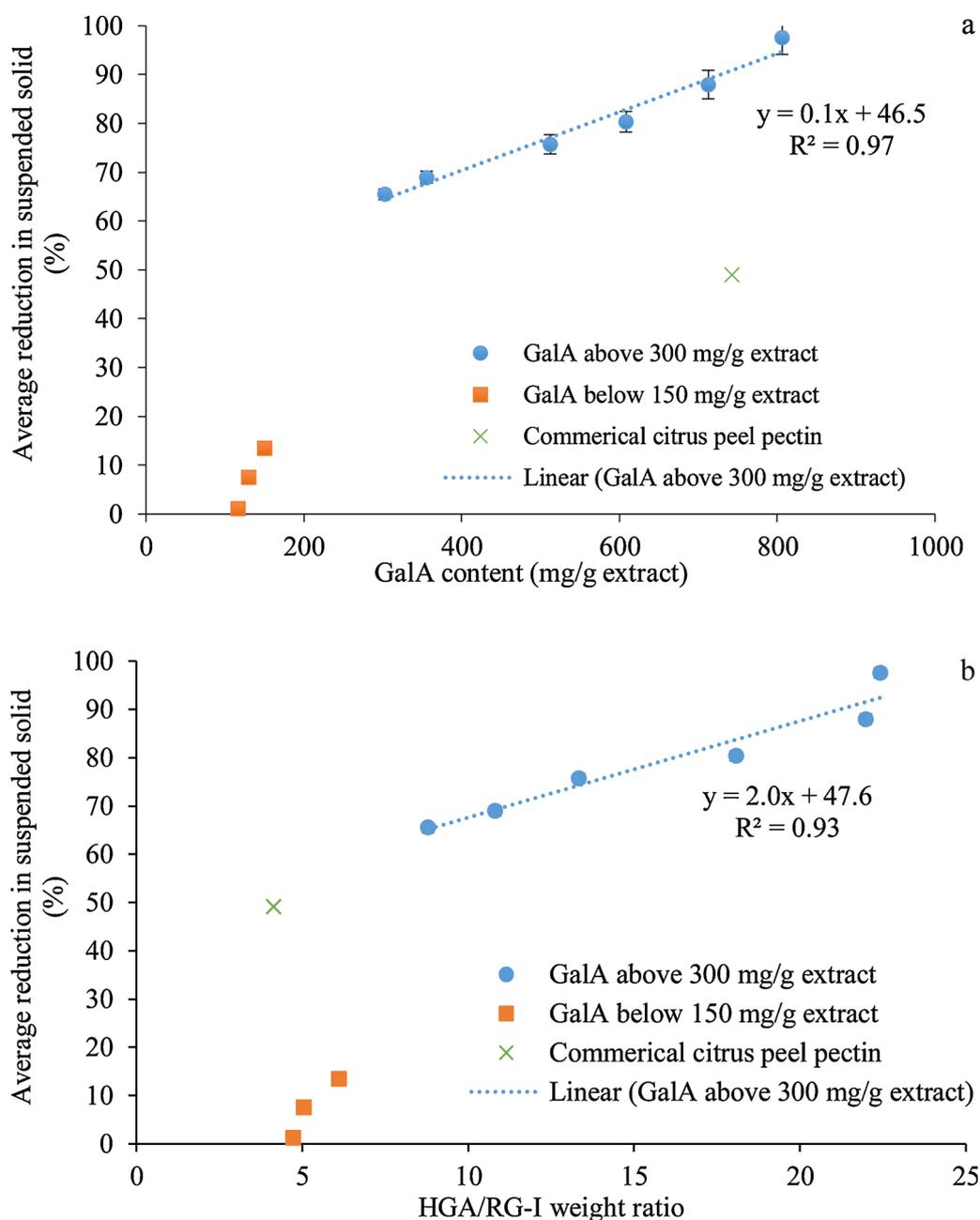


Fig. 4. a. The correlation between extract flocculation ability and GalA content; b. The correlation between extract flocculation ability and HGA/RG-I weight ratio.

finding is opposite to some works that shown the use of strong acids provides high extraction yield and time-saving advantages when other biomass materials were studied, for example citrus pomace (Lim et al., 2012), this present study suggests that the structure of okra pectin may be more fragile or less connected to the cell wall compared to those other biomass materials and therefore the use of strong solvents is not of necessity. In conclusion, this present finding comparing water with other strong aqueous solvents reveals that water not only presents advantages as a green and sustainable solvent, but also offers superior performance of okra pectin extraction.

### 3.2. Chemical and physical properties of bio-flocculants and AIF extracts

Table 1a shows the chemical and physical properties of okra flocculant extracts, AIF and ASF using water, acidic and alkaline solvents. The influence of the three different solvents and the comparisons of mono-sugar content in flocculant extracts, AIF and ASF are discussed

below in detail.

#### 3.2.1. Chemical composition

In terms of GalA compositions, all AIF extracts from water, acidic and alkaline solvents had higher GalA contents compared with flocculant extracts and ASF. This was expected, as GalA content can be used as an indicator of pectin content because GalA is primarily from pectin HGA and RG-I backbones, and pectin is expected to precipitate in alcohol. AIF extracted using water had the highest GalA content of 806.4 mg/g extract, followed by acid AIF extract of 712.8 mg/g extract and alkali AIF extract of 608.2 mg/g extract. The relatively low GalA concentration in AIF extracted under alkaline condition is attributed to the tendency for GalA to degrade under alkaline conditions through  $\beta$ -elimination reaction (Mao et al., 2019). While acidic conditions are known to promote GalA extraction through acid hydrolysis reaction (Mao et al., 2019). It is thought that in the case of okra mucilage, pectin is weakly connected with the cell wall (Anastasakis et al., 2009) such

**Table 1b**  
Chemical and physical properties of raw dried okra biomass and commercial citrus peel pectin.

	GalA. mg/g raw biomass	Rha. mg/g raw biomass	Ara. mg/g raw biomass	Gal. mg/g raw biomass	Glu. mg/g raw biomass	Xyl. mg/g raw biomass	Total sugar mg/g raw biomass	Total RG-I region mg/g raw biomass	HGA/RG-I Weight ratio	DM %	$[\eta]$ mL/g
Raw dried okra	528.3 ± 2.1	9.4 ± 1.5	14.5 ± 0.3	55.5 ± 0.1	212.1 ± 0.2	15.1 ± 0.2	834.9 ± 4.4	79.4 ± 1.9	6.7 ± 0.4	26.2 ± 1.3	/
Commercial citrus peel pectin	742.6 ± 1.9	23.7 ± 1.2	47.3 ± 0.7	108.2 ± 0.9	26.6 ± 0.1	1.5 ± 0.1	949.9 ± 4.6	179.2 ± 2.8	4.1 ± 0.7	45.4 ± 2.7	522 ± 1

that okra pectin is relatively easy to extract, and strong solvents are not necessary. This means that although acid may have promoted the extraction slightly, this benefit was overshadowed by the tendency for strong solvents such as acid and alkali to degrade the extracts (Fraeye et al., 2007).

Similarly to GalA, all AIF extracts from water, acid and alkali had the best Rha, Ara and Gal contents compared to flocculant extracts and ASFs, because these three mono-sugars are the main components of pectin RG-I side chains. AIF extracted using water had the best Rha, Ara and Gal contents of 5.4, 12.1 and 18.9 mg/g extract respectively, followed by alkali AIF extract of 4.8, 11.0 and 17.3 mg/g extract, and acid AIF extract of 3.9, 9.2 and 14.5 mg/g extract. These results can also be shown in terms of the content of total RG-I region, which is the sum of Rha, Ara and Gal. Water AIF has the highest total RG-I region content of 36.4 mg/g extract, followed by alkali AIF of 33.1 mg/g extract and acid AIF of 27.6 mg/g extract. The reason alkali AIF had higher RG-I content than acid AIF is that alkali often promotes the extraction of hairy pectin side chains while acid degrades them through acid hydrolysis reaction (Mao et al., 2019). The reason that water AIF had the higher RG-I content is similar to the reason it had higher GalA content that pectin is weakly connected with the okra cell wall (Anastasakis et al., 2009) such that okra pectin is relatively easy to extract, and strong solvents are not necessary.

Okra AIF extracts using all three solvents have similar Xyl contents, however acid AIF extracts have less Glu content at 87.4 mg/g extract than water AIF at 108.7 mg/g extract and alkali AIF at 109.8 mg/g extract. Glu and Xyl are mainly from small molecular hemicellulose, phenolics, flavonoids that were not purified after AIF precipitation (Wang et al., 2016), and therefore these two sugars would also show in AIF extracts. The reason water AIF had relatively high Glu and Xyl content is similar to the reason why it has highest GalA and RG-I content that water helps to preserve hemicellulose sugars. The reason alkali AIF also have high Glu and Xyl content is that hemicellulose is more soluble in alkaline solutions compared to water and acid solutions (Doner & Hicks, 1997), which leads to more hemicellulose being extracted in alkaline solution. Therefore, although more hemicellulose was degraded in alkali and acid, alkali AIF extract still had higher hemicellulose sugar contents than acid AIF.

Last but not least, in terms of total sugar content, which is the sum of GalA, Rha, Ara, Gal, Glu and Xyl, extracts using water as the solvent had highest total sugar contents followed by extracts using acid and finally alkali. Comparing the three different kinds of extracts, AIF extracts had highest total sugar contents, which was expected as these five mono-sugars are the main components of pectin (Glu and Xyl may be mainly from hemicellulose). Among all extracts, water AIF had the highest total sugar content of 988.6 mg/g extract, suggesting that AIF extracted using water mainly consist of pectin. ASF extracts had the lowest total sugar content, which suggests that although ASF extracts also contain some hemicellulose and pectin, the main component in ASF might be non-structural cell wall component, for example protein and ash.

### 3.2.2. Degree of methylation (DM)

The values of DM of pectin were estimated by calculating the ratio of the molar percentage of methanol to the molar percentage of GalA. Comparing flocculant extracts, AIF and ASF extracts, AIF extracts had relatively lower DM values while ASF had relatively high DM value. For example, water AIF had DM value of 15.9 % compared to 43.3 % from water ASF; similar trends were also found in acid AIF and ASF with DM value of 6.8 % and 77.7 % respectively, as well as in alkaline AIF and ASF of 20.1 % and 87.4 % respectively. This is because DM can be a predictor of extract solubility in water and alcohol (Harding et al., 2017). Extracts with higher DM are highly methylated and thus lower charged, hence they have higher solubility in alcohol than in water, and therefore end up in the ASF fraction. Extracts with lower DM, in the opposite way, are less methylated and thus higher charged, so they

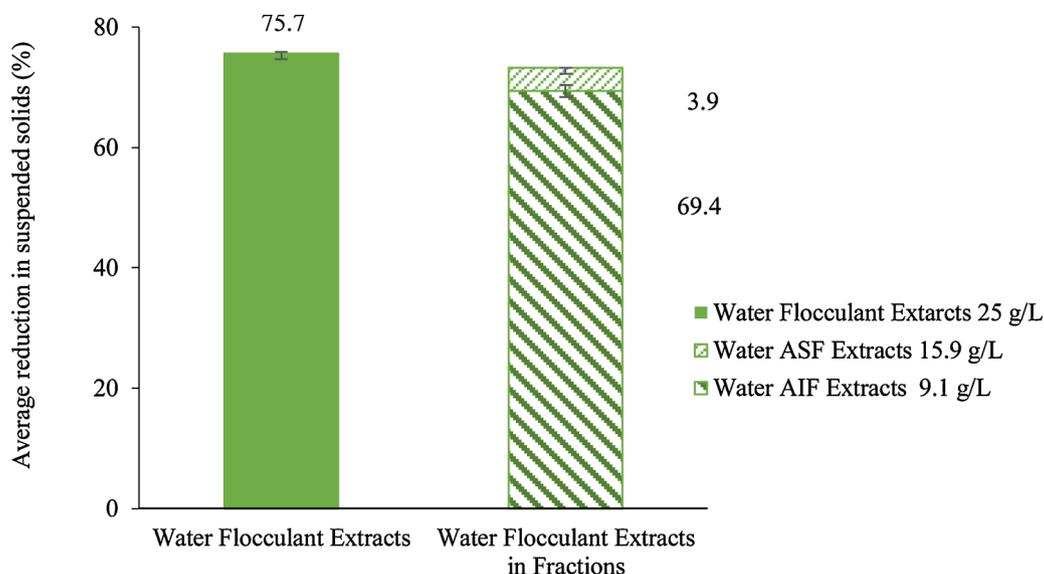


Fig. 5. Flocculation abilities of water flocculant extract and extracts in fractions.

have higher solubility in water but lower solubility in alcohol, and therefore precipitate in alcohol and end up in AIF fraction. Okra flocculant extracts had lower DM values than those of ASF but higher DM than AIF, because they contain both ASF and AIF fractions.

### 3.2.3. Intrinsic viscosity [ $\eta$ ]

The intrinsic viscosity can give information relating to the size and hydrodynamic volume of extracts (Harding 1997). Shown in Table 1a, okra flocculant extracts had much lower intrinsic viscosity than AIF extracts using all three solvents. For example, the intrinsic viscosity of okra flocculant extracts using water was 255 mL/g compared with AIF extracts using water as 1740 mL/g. This is because flocculant extracts contain not only pectin, but also hemicellulose, and non-structural components like ash and protein. When comparing AIF extracts (which are mainly pectin and having similar total pectin content values), lower HGA/RG-I ratios correspond to higher intrinsic viscosities, which suggests higher molecular weight of the extracts. For example, alkali AIF had the highest intrinsic viscosity value of 4890 mL/g and the lowest HGA/RG-I ratio of 18.4. Water AIF extracts had second highest intrinsic viscosity of 1740 mL/g and the second lowest HGA/RG-I ratio of 22.1. In addition, acid AIF extracts had the lowest intrinsic viscosity of 1360 mL/g and the highest HGA/RG-I ratio of 25.8. This is because lower HGA/RG-I ratios correspond to less HGA region and more RG-I region (in this way, HGA/RG-I ratio can also be referred as smoothness index). The molecular weight of RG-I region is usually higher than HGA region due to the presence of numerous side chains in RG-I region, and therefore the RG-I region usually has higher intrinsic viscosity than HGA region. The intrinsic viscosity of ASF extracts were not applicable as they were very close to the solvent buffer, and this is because ASF extracts only have very little polysaccharide contents and therefore very little contribution to the intrinsic viscosity. It needs to be noted that the intrinsic viscosities of okra AIF (1360–4890 mL/g) are relatively larger than some other biomass pectin extracts in the literature review, for example mango extracts (364–1346 mL/g, depending on the extraction condition used) (Koubala et al., 2008), and this suggests that okra pectin extracts have larger molecular structure than those biomasses. The intrinsic viscosity of commercial citrus peel pectin was found to be 522 mL/g, which is consistent with the value (~600 mL/g) found in literature review (Harding et al., 2017).

### 3.3. Flocculating ability in SS removal

#### 3.3.1. The flocculating ability of various okra extracts at 25 g/L

The flocculating abilities of the okra flocculants, okra ASF and AIF extracts were determined using the Jar Test, and the reduction in suspended solid (SS, %) was measured. Results are shown in Fig. 3. Higher reduction in suspended solid (%) corresponds to increased ability of the extract to remove the colloidal particles (in this study, kaolin powder) from wastewater (i.e. better flocculating ability). Between the three kinds of extracts, AIF extract had the highest SS removal, followed by flocculant extract; while ASF extract had relatively low to no flocculating abilities. This suggests that the flocculating property of bio-flocculants extracted from okra is most likely due to the pectin present. Comparing the flocculating ability of three AIF extracts, water AIF had the highest SS removal of 97.6 %, followed by acid AIF of 88.0 % and alkali AIF of 80.4 %. Similarly, comparing the flocculating ability of three flocculant extracts, water flocculant had the highest SS removal of 75.7 %, followed by acid flocculant of 69.0 % and alkali flocculant of 65.5 %. It is not hard to find that the order of values of bio-flocculation ability corresponds directly to the order of values of GalA contents in the extracts. For example, water AIF extracts had the highest bio-flocculation ability because of its highest GalA content of 806.1 mg/g extract.

To further investigate the regions in okra extracts that are responsible for their bio-flocculating ability, Fig. 4a was plotted to show SS removal versus GalA content (that represents the pectin HGA region content). It is shown that using extracts containing more than 300 mg/g extract of GalA, there is a linear relationship between the SS removal and GalA content ( $y = 0.1x + 46.5$ ,  $R^2 = 0.97$ ). This suggests that the region that is predominantly responsible for the flocculating property of okra extracts is the pectin HGA region. Additionally, the way pectin works in flocculation may be best explained through polymer bridging, because pectin HGA regions are long chain polymers that have long loops and tails, and once absorbed can create 'bridging' between particles. Furthermore, increasing the amount of pectin in the extract means more chances for attachment and interactions between absorbed polymer segments and colloidal particles, thus improving the bio-flocculating ability. However, this linear relation between SS removal and GalA content cannot be applied to extracts with GalA contents lower than 150 mg/g extracts, which may be due to the fact that a certain amount of polymers are of necessity for flocculation to occur. For extracts with GalA between 150–300 mg/g, there was no data to determine the relation between SS removal and GalA content. It is noted

that the reason ASF extracts show relatively low to no flocculating ability is due to not only the low pectin content in ASF but also the high DM values of pectins present in the ASF extract. Once the pectin is methylated, it becomes hydrophobic and thus can no longer absorb the particles or create 'bridging'. The effect of excessive amount extracts were not determined in this work, and the only dosage used was 1 mL of 25 g/L extracts, which was the optimal dosage found in previous work of Lee (2017).

To confirm the above linear relation, a commercial citrus peel sample was used. The chemical and physical properties of this commercial citrus peel pectin were found and listed in Table 1b. It has GalA content of 742.6 mg/g extract, which was above 300 mg/g extract and in the range of above found linear relation. However, the SS removal of it at 25 g/L was only 49.0 %. Clearly illustrated in Fig. 4a, commercial citrus peel pectin did not obey the above linear relation found in okra extract. This might be because the pectin polymer needs to have a chain that is long enough to allow the polymer bridging to occur; while commercial citrus peel pectin has a relatively high GalA content, the structure of the polysaccharides in it is different from the structure of okra extract. The linearity of pectin chains can be mathematically represented as HGA/RG-I ratio. Fig. 4b illustrates the correlation between SS removal and HGA/RG-I ratio. At GalA content above 300 mg/g extract, a linear relation of SS removal and HGA/RG-I ratio can be found ( $y = 2.0x + 47.6$ ,  $R^2 = 0.93$ ). Commercial citrus peel pectin was also in line with this linear relationship, confirming that above a GalA threshold of around 300 mg/g, flocculating abilities can be predicted by the HGA/RG-I (or smoothness) ratio of the pectin.

### 3.3.2. The comparisons of flocculating ability of different water extracts

As water extracts showed the best flocculating ability and in order to further investigate the role of pectin in flocculation, the flocculating ability of water flocculant extracts and the relative contribution of AIF and ASF in flocculants were measured and presented in Fig. 5. The flocculant extracts were made into a concentration of 25 g/L, while the concentration of AIF extracts and ASF extracts were calculated based on AIF yields and mass balance so that they were as same as concentrations of these two fractions in the 25 g/L flocculant extracts, which were 9.1 g/L and 15.9 g/L respectively. The dosages of these three extracts in Jar Test were all 1 mL.

The SS removal of water flocculant extracts was 75.7 %, of AIF extracts and ASF extracts were 69.4 % and 3.9 % respectively. It is obvious that most of the flocculation occurred in flocculant extracts was due to the AIF extracts rather than ASF extracts, which again suggests that pectin is the main reason that okra extracts have bio-flocculation ability. The reason that the ASF extracts showed some flocculating ability may be because they also contained some pectin that was not separated from AIF extracts. The sum of SS removal of AIF and ASF extracts was 73.3 %, which was slightly less than the SS removal of water flocculant extracts, and this may be because there was extract lost in the handling sequence of extraction and extracts rehydration as well as in the Jar Test, however the difference was still within the tolerance.

## 4. Conclusion

To summarise, during the sequence extraction method, three kinds of okra extracts were obtained: okra flocculants, ASF and AIF. The optimum treatment condition for extractions using all three solvents was 10 min. time and 90 °C. Flocculant extracts were mostly likely to be both pectin and hemicellulose and other non-structural cell wall components (i.e. protein, ash etc.); AIF should be primarily pectin with some impurified hemicellulose content; while ASF should be primary non-structural cell wall components and hemicellulose with some imprecipitated pectin. Water is the best solvent to use in the case of pectin extraction no matter for pectin HGA or RG-I region extraction, because strong solvents like acid or alkali may quickly degrade the extracts. In addition, flocculant extracts, AIF extracts and ASF extracts from water

all contain higher sugar content than acid or alkali extracts of three different kinds correspondingly. Among all extracts, water AIF had the best total sugar contents as 988 mg/g extract and highest GalA content as 806.1 mg/g extract, suggesting water AIF has the purist pectin content.

In the present study, the bio-flocculating ability of different okra extracts was determined and was found in a linear relationship with GalA content ( $y = 0.1x + 46.5$ ,  $R^2 = 0.97$ , when GalA content > 300 mg/g extract). This suggests that pectin and especially pectin HGA region are the polysaccharides that responsible for okra extracts flocculating ability; and the way pectin works in flocculation may be best explained by polymer bridging mechanisms. When also considering the effect of the structure of pectin, a linear relationship between bio-flocculating ability and HGA/RG-I weight ration was also found ( $y = 2.0x + 47.6$ ,  $R^2 = 0.93$ , when GalA content > 300 mg/g extract), which again suggested HGA was the region for okra extract flocculating ability as a long smooth HGA chain is compulsory for flocculation to happen. This correlation was also validated using commercial citrus peel pectin.

## CRedit authorship contribution statement

**Yujie Mao:** Conceptualization, Methodology, Investigation, Validation, Writing - original draft. **Rachael Millet:** Investigation, Validation. **Chai Siah Lee:** Conceptualization, Methodology. **Gleb Yakubov:** Writing - review & editing, Supervision. **Stephen E. Harding:** Resources, Writing - review & editing, Supervision. **Eleanor Binner:** Conceptualization, Resources, Writing - review & editing, Supervision, Funding acquisition.

## Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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