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The Isolation of taste compounds in *Bekkai lan* (*Albertisia papuana* Becc.) leaves extract using nanofiltration

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Abstract

The purpose of this study is to elucidate the contribution of taste compounds to the flavor in medium alkaline. The alkaline extraction of crude bekkai lan leaves was done at pH 8 while the isolation of taste compounds was done by employing nanofiltration method. The analysis of taste compounds using molecular weight cut-off less than 500 Da was also performed. Alanine, oxalic acid, malic acid, gallic acid, sucrose, fructose, glucuronic acid, Na, K, Mg, Ca, and P were detected in the leaf extract, but no umami compound was detected. The analysis results suggested that the efficiency to obtain the taste compounds from water soluble extract up to fraction 400 Da is 65.66%. The highest decreased mineral to salty compound is Na (54.48%) and the lowest is P (13.87%). The results of this study were useful to better understand the taste compounds obtained from alkaline medium.

Introduction

It is uncommon to treat plant or animal-derived foods using alkali although a high pH can be obtained during a cooking process. Basically, cooking methods at the household scale include boiling, frying, microwave cookery, steaming, sautéing or pan-frying and grilling (Liu and Li, 2000; Bastin, 2000; Yuan *et al.*, 2009). It has been known that cooking can significantly induct the change in chemical composition, bioavailability, and chemopreventive contents. In addition, the method of cooking may affect the obtaining of nutrition and phytochemicals (Sikora, 2008). In this study, the extraction was performed by boiling the material as this method is assumed to be simple, easy, and cheap at a household scale (Bastin, 2000).

One of the factors that may affect the nutrition in cooking process is the acidity of medium (Brandt *et al*., 1984; Girnth-Diamba, 2007). The acidity may affect the availability of nutrition or the formation of color, flavor, and toxin compounds (Brandt *et al*., 1984; Koca *et al.*, 2006; Girnth-Diamba, 2007). As reported by Friedman and Jurgens (2000), the higher pH may be used to recover proteins from cereals and legumes as well as to destruct phenolic compounds. Vegetables will be softer if cooked at pH 10 and conversely, the greater pH will have greater effects in the changes of fiber components (Brandt *et al*., 1984) and colors, while the texture of vegetables will be very green and soft at buffer pH 8 (Girnth-Diamba, 2007).

This study focuses on the extraction of taste compounds under the alkaline condition. Taste compounds in natural products have an MW range of less than 500 Da, so the isolation method using NF is applied to obtain the MWCO of 400 Da and 1000 Da. This method is very applicable for industry and now has become the standard for numerous manufacturing applications (U.S. Department of Energy, 2012). This study is aimed at investigating the determinants of taste compounds especially during the extraction process using boiling method under alkaline condition. The result of this study can be used to understand the effects of alkaline medium in the flavoring.

Materials and Methods

Materials

Bekkai lan leaf powder was prepared as described by Purwayantie *et al.* (2013). The *Albertisia papuana*

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Becc. leaf powder was obtained from local indigenous people, i.e. Dayak Kenyah ethnic group. The leaves were harvested in May 2011 from the secondary forests surrounding Long Le-es village in Busang Subdistrict, East Kutai Regency, East Kalimantan Province, Indonesia. To obtain the leave powder, fresh leaves were sliced, dried under sunlight for 2 days and grounded into powder of 60 mesh in size. The dried leaves and powder samples were stored in the dark at low temperature (-20^{o}C) until used.

Methods

Extraction of water soluble

The extract was prepared from the leaf powder made by the following process: 6 grams of sample were homogenized in 180 ml solution of 0.2 M Tris-HCl buffer pH 8 for 3 minutes by vortex, then boiled (100^{o}C) for 3 minutes too. The mixture was vacuum filtered and the residues were re-extracted with the same buffer as explained in the previous stage. The collected filtrate was referred to as water soluble extract (WSE or alkaline extract), freeze dried, and stored at freezing temperature (-20^{o}C) until used.

Multistep nanofiltration

Water-soluble extracts were fractionated by using the NF membrane method using a 'dead end' filtration system with polyethersulfone (PES) at Membrane Research Centre, Bio separation Laboratory, Faculty of Chemical Engineering, Universitas Diponegoro, Indonesia. The equipment modules consist of plate and frame, 3 liter capacity, 47 mm diameter membrane, and the active surface area of 4 cm^2 with separation mechanism sieving. Worked system: multistep filtration started from microfiltration/MF (PES; the compacting pressure and filtration pressure (<1 and 3bar), NF 30 kDa (PES; 4 and 5bar), NF 10 kDa (PES; 5 and 7.5 bar), NF 1 kDa (PES; 8 and 12 bar) and 400 Da (PES; 12 and 15 bar). PES membrane was obtained from Membrane Research Centre and NADIR, Germany. Permeat was freeze dried and stored at -20°C. The NF permeate produced 1000 Da and 400 Da respectively referred to as fractions of 1 kDa (F-1000) and fractions of 400 Da (F-400).

Chemical analysis

The chemical analysis was performed only on water-soluble extracts, F-1000 and F-400. Total soluble sugar and total acid were measured spectrophotometrically using a method as proposed by Apriyantono *et al*., (1989). Total soluble sugar: 10 mL from those samples were homogenized with 5 ml of 95% ethanol, vortexed and later incubated with 1 ml phenol 5% for 10 minutes, centrifuged at a speed of 3400 rpm for 10 min and then filtered to get the supernatant. A sample (4 mL) was mixed with concentrated 5mL H_2SO_4 stood for 10 minutes and then placed in a water-bath set at 100°C for 15 min. The sample was measured at an absorbance of 490 nm using a UV-Vis spectrophotometers-1601 (Japan). Glucose was used as a standard stock solution to prepare a standard curve for the quantification of total soluble sugar in the sample and expressed as mg g-1 DW of leaves. Total acid: 10 ml from those samples were dissolved in 10 ml aquades. Titrate against 0.1M NaOH used 1% phenolphthalein as the indicator in methanol. The end point was reached when the pink color persisted for thirty seconds. Record the burette volume. The calculation of total acid using citric acid for standard: titrate value x 0.0064 (g/100 ml)

Total salt modified from Winton and Winton (1945): 7 ml from those samples were added 3 ml of 5% Potassium chromate and then titrated with 0.1N $AgNO_3$ to the first permanent appearance of red Ag_2CrO_4. The calculation of total salt using NaCl for standard:

$$\% \text{ NaCl} = \frac{\text{titrate value } AgNO_3 \text{ (ml)} \times 0.1 \text{ N } AgNO_3 \times 58.46}{\text{Weight of samples (g)} \times 1000} \times 100\%$$

Total phenol modified from Farhan *et al.*, (2012): five concentrations from those samples were prepared and then 100 µL were taken from each concentration and mixed with 0.5 mL of Folin–Ciocalteau reagent (1/10 dilution) and 1.5 mL of Na_2CO_3 2% (w/v). The blend was incubated in the dark at room temperature for 15 min. The absorbance of blue-colored solution of all samples was measured at 765 nm using UV-Vis spectrophotometers-1601 (Japan). The results were expressed in mg of gallic acid equivalent (GAE) per g of dry weight of leave powders as a percent total phenol.

Total amino acid (Khokhani *et al*., 2012): 10 ml of those samples were homogenated with 5 to 10 ml of ethanol (80%), filtered and centrifuged. To 0.2, 0.4, 0.6, 0.8, 1.0 ml of extracted 1.0 ml ninhydrin solution was added. 2 ml of distilled water contents test tube were heated in the boiling water bath for 20 minutes to which 5 ml of the 80% ethanol were added and contents were mixed. After 15 min, the intensity of the purple color against a reagent blank on a spectrophotometer at 570 nm was recorded. The color is stable for 1 hour. The reagent blank as mentioned above by taking 0.1 ml of 80% ethanol instead of the extract was prepared. Construction of a calibration curve: 50 mg aspartic acid in 50 ml distilled water was dissolved in volumetric flask. 10 ml of this stock standard was taken and diluted to 100 ml in another volumetric flask for working

standard solution. Procedure for the sample was performed and O.D. was measured using UV-Vis spectrophotometers (spectronix 200+). Standard curve using absorbance versus concentration was drawn to find out the total free amino acids in the sample and to express as percentage equivalent of aspartic acid in given sample.

The profile of free amino acid (FAA) was analyzed as described modification from Jork et al. (1990). 4 ml from those samples were centrifuged for 2 min and 25 µl of supernatant were mixed with 300 µl of OPA (o-Phthaldialdehyde) solution. The mixture was vortexed for 1 min and 20 µl aliquot was injected and analyzed using HPLC (SHIMADZU LC 10) with a Licrospher 100 RP 18 (5 µm) and a 125 x 4 mm column. The separation of OPA-derivatives was performed using a mobile phase consisting of methanol, 50mM Na-acetate, THF (2:9:2) at pH 6.8 as solvent A and 65% methanol as solvent B. The gradient elution program was held at 100% of A for 0.1 min, ramped at 100% of B for 45 min and stopped at 50 minutes with a flow rate of 1 ml/min. Detection was performed using a fluorescence Shimadzu RF-138 set at 360nm (Ex) and 460nm (Em). Each FAA was identified by using the authentic standard (Sigma-Aldrich) and quantified by the calibration curve of the authentic compound (external standard method).

The profile of 5'-nucleotides was analyzed as described modification from Huang *et al*. (2006). 10 ml from those samples were filtered through 0.45µm filters and 20 µl aliquot were analyzed using the HPLC system consisting of a HPLC Waters E2695 automatic sampler, a Waters SM7 injector, a 20 µl sample loop, a sunfire C18 column (4.6 x 125 mm, 5µm), and a Waters 2489 uv/vis detector. The mobile phase was 500 mmol/L KH_2PO_4/H_3PO_4, pH 4.3 at a flow rate of 1 ml/min and UV detection at 254 nm. Each 5'-nucleotide was identified and quantified by using the authentic standard of 5'-nucleotide: IMP and AMP (Sigma-Aldrich), GMP (Ajinomoto Int, Japan) by the calibration curve of the authentic compound (external standard method).

The profile of acid compounds was analyzed as described modification from Kowalski *et al*., (2007): 10 ml from those samples were vortexed for 2 min, filtered through 0.45µm filters and 20 µl aliquot was analyzed using the HPLC. The HPLC system consisted of a SHIMADZU LC 10 and a 300 x 7.8 mm column. The mobile phase was 20mM KH2PO4 (pH 2.5) at a flow rate of 0.5 ml/min, pump pressure of 344 psi at 50°C condition (isocratic), detected by UV at 226 nm. Each organic acid was identified and quantified by using the authentic standard of malic and oxalic acid (Sigma-Aldrich), by the calibration curve of the authentic compound (external standard method).

The profile of phenolic acid compounds was analyzed as described modification from Truong *et al*. (2007): 10 ml from those samples were vortexed for 2 min, filtered through 0.45µm filters and 20 µl aliquot prior to HPLC. The HPLC system consisted of a SHIMADZU LC 10 and separation was achieved on a 125 × 4 mm, Licrospher 100RP (5µm) column with a UV detector. The elution solvents were A (0.1% v/v formic acid in water) and B (100% methanol) at a flow rate of 0.5 ml/min, pump pressure of 344 psi at 50oC condition. Spectral data from 200 to 600 nm were recorded and the phenolic chromatograms were monitored at 280 and 326 nm. For the isocratic method, the elution was carried out for 20 min with 60% A and 40% B. Each phenolic acid was identified and quantified by using the authentic standard of gallic acid (Sigma-Aldrich), by the calibration curve of the authentic compound (external standard method).

The profile of soluble sugar compounds (BioRad, 1994): 10 ml from those samples were vortexed for 2 min and filtered through filter 45µm and 20 µl aliquot prior to HPLC. The HPLC System was equipped with KNAUER (Metacarb 87C), and separation was achieved on a 300 x 7,8 mm, eluent flow rate of 0.7 mL/min at 65°C condition. The elution solvents were aquabides with isocratic elution. Each soluble sugar was identified and quantified by using the authentic standard of glucose and sucrose (Sigma-Aldrich), by the calibration curve of the authentic compound (external standard method).

The mineral contents of samples such as K, Na, P, Mg, and Ca were determined using atomic absorption spectroscopy (AAS) (Balai Penelitian dan Pengembangan Pertanian, 2005). Calculation of P: 5 ml from those samples and P standard were put into test tubes and added by 1 ml dye reagent of P, homogenated and stood for 30 minutes. The solution of P was calculated spectrophotometrically at 693 nm. Calculation of K, Ca, and Mg: 1 ml from those samples and K, Ca, Mg standard were put into tubes and added by 9 ml of 0.25% La solution, then homogenated. The calculation of Ca and Mg was done using AAS while K was measured using Flamephotometer with standard series as a comparison. Calculation of Fe and Zn: 1 ml from those samples and standard series of Fe and Zn were put into the tubes and added by 9 ml of deionization water and shaken (diluted of 10x) meanwhile, Fe and Zn solution were directly measured using AAS with the standard series as a comparison.

Statistical analysis

The resulting data were tabulated and analyzed descriptively.

Results and Discussion

Crude of taste compounds

The content of total sugar, total acid, total amino acid, total phenol and total salt in crude (water soluble extract) are showed in Figure 1. The content of total amino acid (6.19%) is higher than others, while the total phenol is only produced by less than 1%. The results presented in Figure 1 shows that the content crude of chemical compounds was associated to the taste compounds in alkaline medium was obtained 17.13%. This is an initial identification the crude of the taste compounds as total sugar, total acid, total salt, total amino acid and total phenol. Generally, there are five classes of basic taste that can be distinguished based on their structures. According to Mulay (2012), there is a correlation between the chemical structures and the taste compounds. Acid compounds are primarily associated with acid taste (Beatty and Cragg, 1935); halide salt is usually associated with salty taste (Murphy, 1981); bitter taste compounds may provide a defense against predators by making the plant unpalatable e.g. phenol, alkaloid, terpene (Drewnoski and Gomez-Carneros, 2000). Sweetness is commonly related to soluble sugars (Gomez, 2002) and amino acids (Chen, 1986).

However, the result of this crude of taste compounds cannot be claimed yet to contribute to the taste since this data still reflects rough calculation. The analysis of the crude was done based on the % weight as the preliminary study so that it could be identified by using HPLC and AAS to further evaluate the contribution of each taste compounds.

Profile of taste compounds

In order to further investigate the crude, the concentration of taste compounds was also measured by HPLC and AAS. Basically, taste compounds are classified into five groups: sweet, acid, bitter, salty, and umami but in this profile results, umami compounds were not detected. The results presented in Figure 2 shows that 12 taste compounds are detected in alkaline medium are. The sweet compounds detected are soluble sugars (sucrose; MW 342.30Da and fructose; MW 180.16Da). In addition, the group of alanine amino acid (MW 89.09Da) is also detected; this contributes to the sweetness. The compounds of the organic acids detected are malic

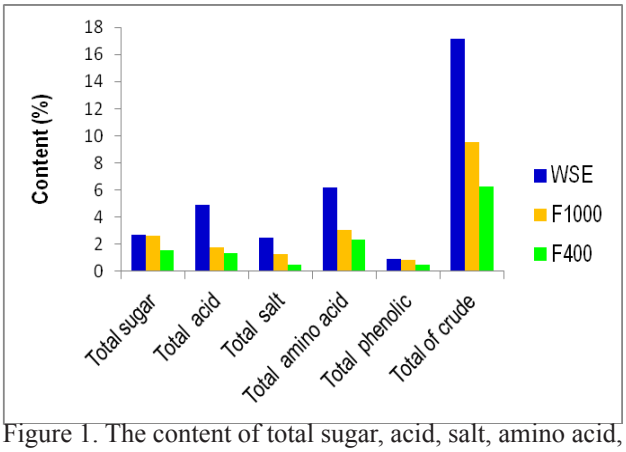

Figure 1. The content of total sugar, acid, salt, amino acid, phenolic and total of crude of chemical compounds were associated to the taste compounds on WSE, F-1000 and F-400

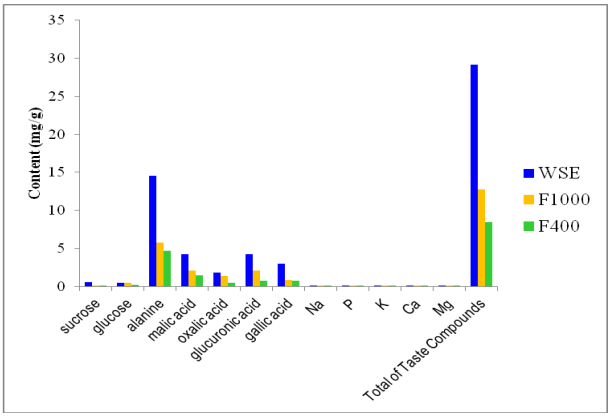

Figure 2. The content of taste compounds during NF on WSE, F-1000 and F-400

acid (MW 134.09Da), oxalic acid (MW 90.03Da), and glucuronic acid (MW 94.1394Da). Only one bitter compound is detected by HPLC, gallic acid (MW 170.12Da). Minerals which contribute to salty are analyzed include Na, K, Ca, P and Mg. The higher taste compounds obtained from the F-400 was alanine (4.69 mg/g) and malic acid (2.05 mg/g) while others are less than 1mg/g. In addition, the total of taste compounds is obtained in F-400 only 8.99 mg/g (0.89%).

Generally, sweet, bitter and acid compounds can be extracted in alkaline medium. Sucrose in alkaline medium was reported by Shaw *et al*. (1969). Under alkaline condition, sucrose is considered relatively unreactive because it lacks unsubtituted hemiacetal grouping in reducing disaccharides (Hassid and Ballou, 1957). However, Browne and Zerban (1941) noted that sucrose is slowly, but steadly, hydrolyzed by strongly alkaline copper reagents and thus can produce a reducing effect on these reagents. Krygier *et al*. (1982); Naczk and Shahidi (2004); Devanand and Pastor-Corrales (2006) reported that phenolic acids and esters were released by alkaline medium. Meanwhile, a report by Attia *et al.* (2005) suggested

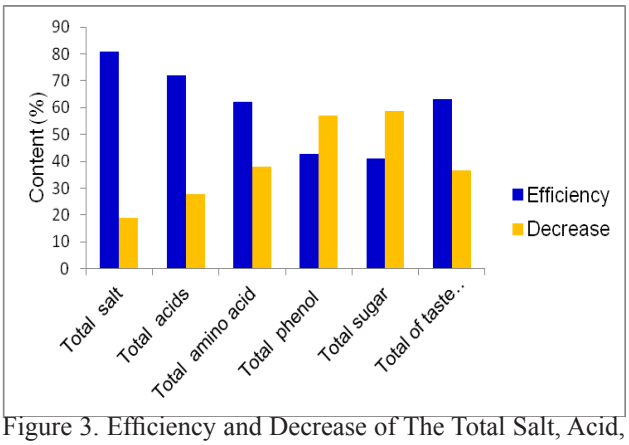

Figure 3. Efficiency and Decrease of The Total Salt, Acid, Amino acid, Phenolic, Sugar and Total Crude of Taste Compounds from WSE up to F-400

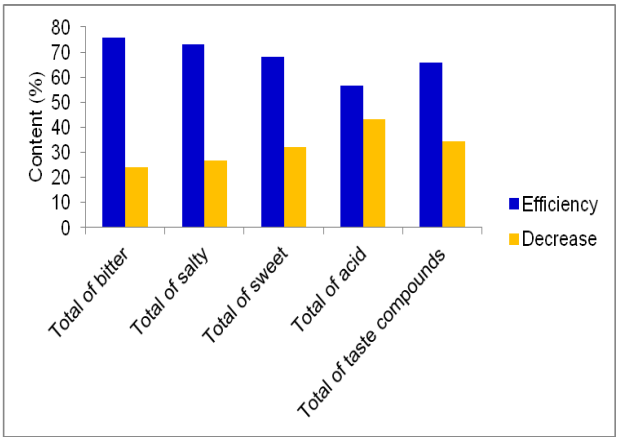

Figure.4. Efficiency and Decrease of The Total of Bitter, Salty, Sweet, Acid and Total of Taste Compounds (from WSE up to F-400)

that the most efficient extraction of organic acids can be performed by alkaline medium. Therefore, sucrose, glucose, gallic acid, malic acid, oxalic acid, glucuronic acid are performed by alkaline extraction.

Minerals P and Ca are found to be the most contribution minerals during filtration membrane (Figure 5), both compounds are higher than Na. As reported by Engel *et al*. (2000), the saltiness derived from goat cheese is also produced from the cations Na, K, Ca, and Mg. Meanwhile, minerals K and Ca have been also reported to have contribution to the taste of seaweed (Dermiki and Methven, 2007).

Nanofiltration (NF)

Membrane filtration method is used to obtain the target compound which does not directly involve organic solvents which are dangerous for humans. Recently, this method has been applied in industries because it has a number of advantages, i.e. the product materials are safety from organic solvent contamination and able to avoid damage or hydrolysis during handling and do not involve heat. Since the target is to get more than one compound and have

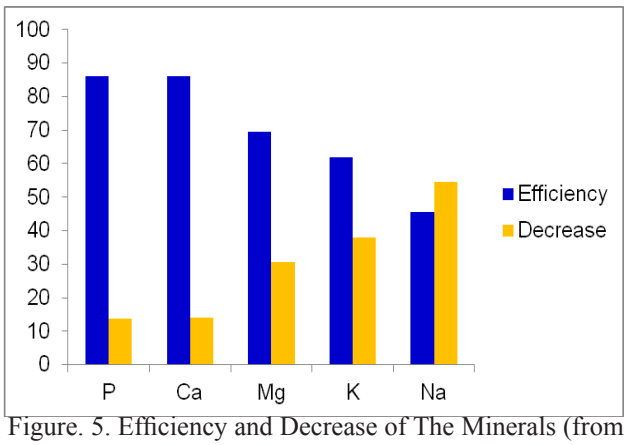

Figure. 5. Efficiency and Decrease of The Minerals (from WSE up to F-400)

a specific molecule weight of less than 500Da, this method is considered the most appropriate.

Data presented in Figure 3 shows the efficiency of total crude of chemical compounds associated with taste compounds during filtration membrane (from water soluble extract (wse) up to 400Da) obtained 63.30% and the decrease 36.70%, but when identified by using HPLC and AAS in Figure 4 shows the efficiency of the total of taste compounds obtained 65.66% and the decrease 34.34%. From Figure 3 and 4 conclude that the chemical analysis of the cude (total sugar, acid, amino acid, phenol and salt) could be used for initial analysis to taste compounds. After identification by HPLC and AAS, the higher efficiency to obtain the taste compounds from the bitter (75.92) and the lower is acid compounds (56.76). The total salty compound obtained from wse up to 400 Da is 73.25% and the higher decresed from Na (54.48%) and the lower is P (13.87%) (Figure 5).

Figure 1 shows that the total crude of chemical compounds is associated with taste compounds (6.29%) in F-400, while in Figure 2 shows the total of taste compounds in F-400 obtained 8.99 mg/g (≈ 0.89%). Therefore, from the 6.29% of total chemical compounds considered as the crude of taste compounds, only 0.89% of taste compounds are identified from the fraction of 400 Dalton. Since the fraction of 1000 and 400 Dalton is obtained 0.89-1.49% while the crude is only 2.62%, the fraction of 5 kDa would be higher obtained in the taste compounds. In this research, the analysis of the fraction of 5 kDa was not performed, thus in the future, to obtain the taste compounds using NF, it should be done only up to MWCO of 5 kDa. This is to avoid the higher loosing of taste compounds in alkaline medium.

Generally, the decrease during membrane filtration is caused by a phenomenon known as 'fouling' (Cheryan, 1986). According to Verliefde (2008), the result of NF or decrease in all types of membrane filtration is naturally unavoidable. At each

step of the membrane filtration, there are always a number of compounds that are not able to pass through the membrane due to the pore size or because the permeation only has compounds corresponding to the MWCO. Furthermore, Cheryan (1986) suggests that fouling is the accumulation of deposits and submicron particles on the membrane surface and or crystallization and precipitation of small solutes on the membrane pore. In addition, fouling may occur because the specific results of physical and chemical interaction from various solids are dissolved on the membrane (Hoek, 2002). Consequently, the fouling occurs during membrane filtration which is strongly influenced by the characteristics of material test, chemical properties, and membrane-solute interaction. Fouling phenomenon always take places on the materials of biological fluid or foods (Hallstrom, 1981). According to Hoek (2002), the phenomenon of fouling almost always occurs in the 'dead end' system. In this system, the pore is easily covered by the vertical stream of feed solution. Almost all chemical compounds in the filtered material create a fouling phenomenon. The main ingredients whose frequencies always cause fouling are proteins, lipids, salts (Ca, P), and microorganisms (Cheryan, 1989). In this research, the major minerals decreasing during NF are Na and K, thus contributing to the fouling (Figure 5)

In addition to the types of membrane (Ami and Cho, 1999; Fan *et al.*, 2001), many other factors can affect the results of the target compounds by NF including, as reported by Kabsch-Korbutowicz (2008), natural organic matter characteristics (Laine *et al*., 2002); pH and solute concentration (Jarusutthirak *et al*., 2007). In this research, these factors are not analyzed since this is a preliminary exploration. Therefore, in the future, in-depth researches on a variety of those factors are necessary to conduct to obtain efficient and economical taste compounds.

Conclusion

The study on the isolation of taste compounds in bekkai lan (*Albertisia papuana* Becc.) leaf extract especially in the alkaline medium using nanofiltration is a novel study. In the extraction condition under the 0.2 M Tris-HCl buffer at pH 8 heated for 3 minutes, all taste compounds were obtained except umami. The sweet compounds of bekkai lan leaf extract included sucrose, fructose, and alanine. Meanwhile, the minerals which contributed to the salty compounds of bekkai lan leaves extract included K, P, Mg, Na, and Ca. In addition, the acids were obtained from malic, oxalic, and glucuronic acid while the bitter was only gained from gallic acid.

The efficiency to obtain the taste compounds from water soluble extract up to fraction 400 Da was 65.66 % and the content of total taste compounds detected was only 0.89% on fraction of 400 Dalton. So, it is not recommended to perform an isolation taste compounds on MWCO of less than 500 Dalton, but to avoid the loss of taste compounds, it should be done only up to MWCO of 5 kDa. Further studies are needed to determine the effects of membrane types in obtaining the higher taste compounds in bekkai lan leaf. In the future, such studies will be essential in the flavoring industries applying NF method.

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