Extraction of Precursors for Polyurethane Foam Production

Saartjie Gouws[1], Sanette Marx[1], Idan Chiyanzu[1], and Japie Mienie[2]

Abstract**—** *Amaranthus cruentus* stems have great potential for use as feedstock in the synthesis of bio-based products (such as polyurethane foam) due to the abundance of amino acids present in the plant. In order to utilize these compounds they need to be extracted from the stems of the plant in the form of proteins. This is followed by a protein hydrolysis step to obtain the amino acids. The main objective of this study was to develop an effective and environmentally friendly method to extract proteins from amaranth stems. Microwave-assisted subcritical water hydrolysis to extract proteins from the stems was performed at 240, 260 and 280°C in dilute acid and alkaline solutions. The proteins were hydrolyzed to amino acids using Neutrase® 0.8 L enzyme at its optimal performance conditions. The method developed yielded 19 primary amino acids as well as some of their derivatives. A higher concentration was observed in the dilute acid solutions due the acid's ability to prevent polymerization of the amino acids.

Keywords**—***Amaranthus cruentus*, amino acid, enzymatic hydrolysis, protein extraction, subcritical water hydrolysis.

I. Introduction

WE are living in an era where the consumption of energy is ever increasing. Over the past few years the world has become more aware of its energy source limitations and the concerns around climate changes. Environmental conservation has become crucial. For this reason the transition to a bio-based production system in order to achieve economic and environmental sustainability has commenced [1]. By the end of the next century several of the current petroleum-based products may be replaced by more economical, superior and renewable materials [2].

Biomass is an important feedstock for the production of renewable products. Ethanol has been produced through the conversion of lignocellulosic resources [3]. Plant oils have been utilized for the production of various oil-based chemicals and materials such as polyurethanes [4], biodiesel fuel [5] and lubricants [6]. The sugar and oil content of biomass are attractive raw materials to prepare chemical products, but the value of the proteins present in plant materials should not be overlooked. Protein from biomass can be used as feedstock for producing various bio-based products including pharmaceuticals, plastic food packaging and fabrics [7].

Amino acids present in the proteins have many uses such as flavor enhancers (artificial sweetener) and additives in feed and pharmaceuticals [8]. Research is being conducted for using amino acids to prepare chemicals which were conventionally produced by the petrochemical industry. Researchers have used amino acids to produce bio-based isocyanate, a main component in the production of polyurethane foam (PUF) [9]. Other authors surmise that if all amino acids can be used as feedstock in the chemical industry, it could equal the replacement of approximately 2000-3000 PJ of fossil materials [10].

Amaranthus cruentus (Amaranth) is an attractive biomass due to its high protein content, its ability to adapt to different environments and to grow vigorously. It also displays a high resistance to drought, heat and pests [11]. The plant is viewed as a useful additive in foods due to its medicinal benefits and high nutritious value [12]. Ethical issues surrounding the use of edible biomass for the production of bio-based products emphasizes research in the utilization of the non-edible parts of plants such as the stems. In order to establish a complete bio-refinery process of biomass (such as amaranth), the main focus should be on the extraction and hydrolysis of proteins to amino acids. A variety of protein extraction methods are available based on their physiochemical and structural properties [13]. Characteristics of the natural material from which the proteins are extracted also play an important part in the selection of an extraction technique [14]. As reported from previous literature, protein extraction is mainly performed in solid-liquid phases. The solvents most often used are organic compounds such as acetic acid [15], aqueous isopropanol [16] or inorganic acids and bases like hydrochloric acid and sodium hydroxide respectively [17].

The need for a rapid and environmentally friendly protein extraction method has led researchers to develop new methods such as microwave assisted extraction (MAE) [18], ultrasound-assisted extraction [19], supercritical fluid extraction [20] and subcritical water extraction (SWE) [21]. These methods were derived from classical methods i.e.

Saartjie Gouws[1] is with the School of Chemical and Minerals Engineering, North West University (Potchefstroom Campus), Potchefstroom, South Africa.

Sannette Marx[1] is with the School of Chemical and Minerals Engineering, North West University (Potchefstroom Campus), Potchefstroom, South Africa.

Idan Chiyanzu[1] is with is with the School of Chemical and Minerals Engineering, North West University (Potchefstroom Campus), Potchefstroom, South Africa.

Japie Mienie[2] is with the School of Physical and Chemical Sciences, North West University (Potchefstroom Campus), Potchefstroom, South Africa.

chemical (alkali/acid hydrolysis), biological (enzyme hydrolysis) and physical (microwave and ultrasonic) extraction.

In recent years the use of water as an extraction solvent has become popular and desirable. It is inexpensive and environmentally friendly although its use is restricted by its poor extraction efficiency for organic compounds [22]. It has however been established that water has unique solvation properties which may be modified by changing the temperature [23]. It is possible to manipulate the physical and chemical properties of water in a sealed system by controlling the temperature and pressure. Water is retained in a liquid phase even at temperatures above its atmospheric boiling point by applying a pressure greater than its vapor pressure at the reaction temperature. The energy used for vaporization of liquid water (latent heat of vaporization 2.26 MJ/kg) is avoided [24]. An example of this is controlling water at subcritical conditions.

At subcritical conditions (100-374°C) the polarity, surface tension, dielectric constant and viscosity of water is lowered considerably compared to water at ambient conditions, thereby changing its chemical properties to mimic those of organic solvents [22].

Authors compared alkali hydrolysis and subcritical water hydrolysis of protein from de-oiled rice bran [25]. The study showed that the protein yield for the subcritical water hydrolysis was considerably higher than the yield obtained by the alkali method.

A different study showed that flash hydrolysis with a short residence time (10s) under subcritical water medium extracts proteins from microalgae (*Senedesmus sp.*) effectively [24].

The aforementioned studies have made use of batch or continuous reactors for subcritical water hydrolysis. The novelty of this study lies in the use of an industrial microwave to perform protein extraction from amaranth stems by subcritical water hydrolysis. Amaranth is a rather under exploited plant however it is of promising economic value due to its various uses [26]. The protein content of the non-edible stems have not previously been studied in detail and hold a great possibility for use as a raw material to prepare renewable products if extracted successfully.

The effect of temperature changes as well as dilute hydrochloric acid (HCl) and sodium hydroxide (NaOH) concentrations on protein yield during the extraction was investigated. The hydrolyzate (liquid fraction) was analyzed for proteins. Amino acids were obtained through enzymatic hydrolysis.

This study aims to provide a more effective method for protein extraction which will allow the preparation of amino acids as bio-chemicals to be more economical. It further aims to successfully utilize amaranth stems as a source for amino acids. This will certainly contribute to the effective production of better-performing renewable products and the transition towards a bio-based economy.

II. Materials and Methods

A. Raw materials

The stems of *Amaranthus cruentus* cultivated in Potchefstroom at the North West University (S26º41´26´´ E27º05´36´´) were used. The stems were milled and sieved. Particles smaller than 1.5 mm were retained. The milled amaranth stems were then placed in sealed plastic bags and stored under ambient conditions until it was used for protein extraction.

B. Protein extraction

Protein extraction by subcritical water hydrolysis was performed in an industrial microwave (Multiwave PRO, rotor 8NXQ80 from Anton Paar®). The amaranth powder (3 g) was weighed into the capped hydrolysis tubes (80 mL). Next, 27 mL of solution was added to the tubes. The solutions were made up of NaOH and HCl respectively with deionized water in concentrations of 0.5, 1, 1.5 and 2 g/L. The tubes were purged with nitrogen for 1 minute [27]. The extraction was performed at 3 different temperatures i.e. 240°C, 260°C and 280°C. The extraction program of the Multiwave PRO was used and set to hold the temperature for 10 seconds.

After extraction, the product was collected and filtered with a vacuum filter using a 1.0 µm filter paper (Grade 0393, ACE). The solid fraction was recovered and stored in a sealed petri dish at ambient conditions. Finally, the protein content of the liquid fraction was determined by using the Bradford method. The liquid was stored at 4°C.

C. Enzymatic hydrolysis of protein

The commercial enzyme used in this study was the protease, Neutrase® 0.8 L which was supplied by Novozymes A/S, Denmark.

The method published by Damrongsakkul and co-workers [28] was used for this step. The optimum mass ratio of neutrase enzyme to amaranth protein of 0.075:100 was used for all experiments. The pH of the solution was adjusted to 6. The enzyme was added and the solution mixed in an incubator at an agitation of 75 rpm at 40°C for 3 hours. The enzymes in the samples were deactivated by rapidly heating the samples in an oven to 90°C for 15 min. The samples were then centrifuged for 15 min before filtering with a vacuum filter using a 0.45 µm nylon syringe filter. These samples were stored at 4°C until amino acid analysis.

D. Analytical methods

1) Determination of protein

The protein content was determined with the Bradford protein assay provided by Sigma-Aldrich®. The assay was done directly into a cuvette by adding 1.5 ml of Bradford Reagent (B6916 from Sigma-Aldrich®) to 0.05 ml of the sample. The absorbance was read at 595 nm on a UV mini-1240 SHIMADZU spectrophotometer.

2) Amino acid analysis

a)Thin layer chromatography

Thin layer chromatography analysis was performed in order to identify the amino acids present in the samples. This analysis works on the principle of separating amino acids based on their different affinity. A TLC Alufoline plate was used as the stationary phase. The mobile phase was a mixture of n-butanol, acetone, water and glacial acetic acid in a ratio of 35:35:20:10. A 1.5 cm line was drawn from the bottom of the TLC plate. On this line twelve 1 cm lines where spaced out evenly. 7 uL of different samples were then applied on each line by using a Hamilton syringe while continuously blow drying the plate. 3 uL of a standard amino acid mixture (AA-S-18 from Sigma-Aldrich®) was applied in the middle of the plate. 100 mL of the mobile phase was prepared and poured into a TLC tank until the solution was 1cm from the tank bottom. The TLC plate was then inserted into the tank and the tank was closed. The mobile phase was allowed to run to the top of the plate. The plate was removed and blow dried for 10 minutes after which it was inserted back into the tank to allow the mobile phase to run through the plate a second time. Thereafter the plate was removed and blow dried. The plate was then immersed in a ninhydrin solution in order to color the amino acids and make them visible on the plate. The solution contained 1250 mg ninhydrin, 50 mg isatin, 5 mL 2,6-lutidine 99% and 500 mL acetone. Afterwards the TLC plate was allowed to dry for 15 minutes at 45°C. The amino acids then became visible and were compared to the standard amino acids in order to identify them. This was done by prior knowledge of the retention factors of the various amino acids in the standard.

b) GC-MS analyses

Further amino acid detection and quantification was done on the samples identified to be the most promising from the TLC analysis. This was done by making use of the EZ:faast™ Amino Acid Analysis Kit and a Hewlett Packard HP6890 series GC system with an Agilent 5973N Mass selective detector. The GC system was fitted with a Phenomenex Zebron EZ-AAA amino acid column (part number CG0-7169) as well as a gas clean filter system from Agilent Technologies (part number CP17973). The analysis was carried out with 1.1 mL/min constant flow of helium as the carrier gas. A 2 µL sample was injected in split-less mode. The injector temperature was 250°C. The oven's initial temperature was 110°C and was increased to 320°C at a rate of 30°C/min. The MS source was set to 240°C and the MS quadrupole to 150°C. The scan range of the MSD was 45-450 m/z.

III. Results and Discussion

A. Protein extraction from amaranth

1) Effect of temperature

Subcritical water hydrolysis was performed at 240, 260 and 280°C respectively. The study showed that the temperature affected the protein yield. The highest amount of protein was extracted at 240°C. The protein yields decreased with increasing temperature as seen in Fig. 1. This can be attributed to the partial degradation of proteins which have been observed at higher temperatures [29].

2) Effect of acidic/alkaline solutions

The protein extraction was performed under various concentrations of dilute acid and alkaline solutions. The amount of protein extracted was slightly higher in the dilute acid samples. Some authors have observed that extracting protein under alkaline conditions at high temperatures increases the degradation of the proteins in solution [17].

The temperature however, had a more significant influence on the amount of protein extracted in comparison with the different concentrations of alkaline and acidic solutions as can be seen in Fig. 1.

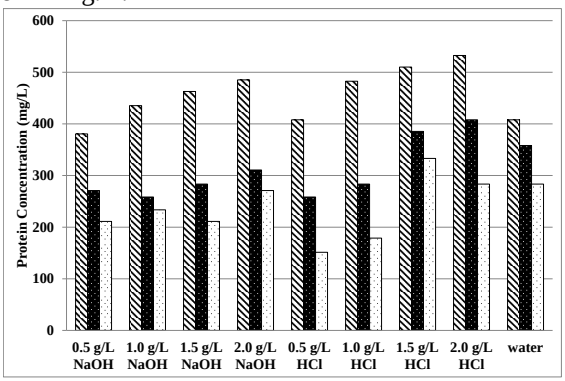

Fig. 1: Influence of acidic/alkaline concentrations and temperature on total amount of protein extracted (mg/L) (▨–240°C ■ – 260°C □ – 280°C).

B. Amino acid composition of amaranth

1) Effect of acidic/alkaline solutions

As discussed previously the samples were prepared in water as well as dilute concentrations of HCl and NaOH. After protein extraction and enzymatic hydrolysis the hydrolyzate was analyzed for amino acids on a TLC plate. A typical TLC plate showing amino acids hydrolyzed from extracted proteins is shown in Fig. 2.

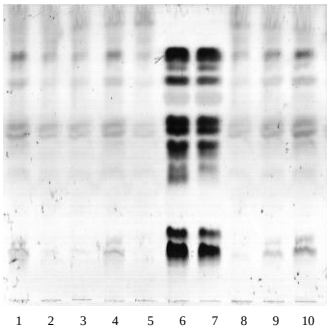

Fig. 2: TLC plate showing amino acids at a temperature of 240°C and various alkaline and acidic solutions.

Samples 1, 4, 9 and 10 were hydrolyzed from proteins extracted with 0.5, 1, 1.5 and 2 g/L HCl respectively. Samples 2, 3, 5 and 8 were hydrolyzed from proteins extracted with 0.5, 1, 1.5 and 2 g/L NaOH respectively. Sample 6 and 7 represents the standard amino acid solution.

From the TLC plates it was observed that the amount of amino acids increased with increasing HCl concentration but decreased with increasing NaOH concentration. This indicates that there was a higher variety of amino acids present in the samples under acidic conditions in comparison to those under alkaline conditions. This can be explained when considering that some amino acids such as serine, arginine, threonine and cysteine are destroyed during alkaline hydrolysis [30].

The samples containing the alkaline solutions had a dark brown appearance whereas the acidic samples were light yellow and clearer. From these observations it is surmised that the acid aids in preventing polymerization of the amino acids which makes the liquid clearer. It was also noted that once the samples were removed from the industrial microwave the solutions became darker with time as more of the amino acids polymerized.

The concentration of tryptophane was quite low (Fig. 3-5). This can be explained when considering that tryptophane is stable only under basic conditions [30]. It is the only amino acid which would be more abundant in the samples containing NaOH. The 2g/L HCl solution was identified as the acidic concentration for which the highest quantity and variety of amino acids were obtained.

2) Effect of temperature on amino acid concentrations

The samples containing 2g/L HCl were analyzed further on a GC-MS. The amino acid compositions of these samples at temperatures of 240, 260 and 280°C are shown in Figures 3 to 5 respectively.

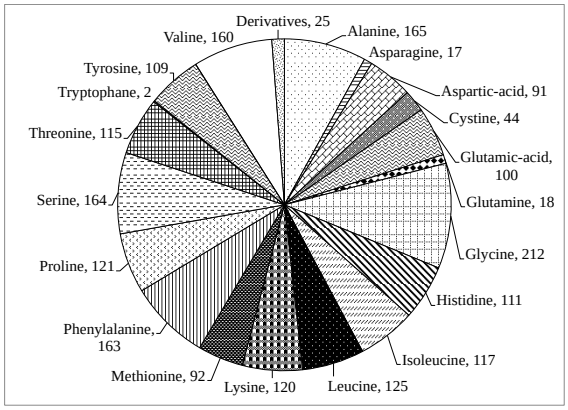

Fig. 3 Amino acid composition (µmol/L) at 240°C

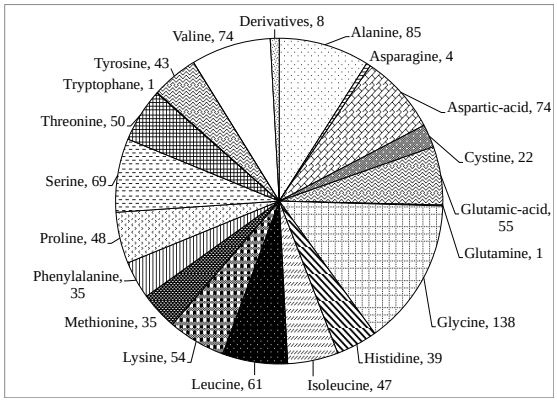

Fig. 4 Amino acid composition (µmol/L) at 260°C.

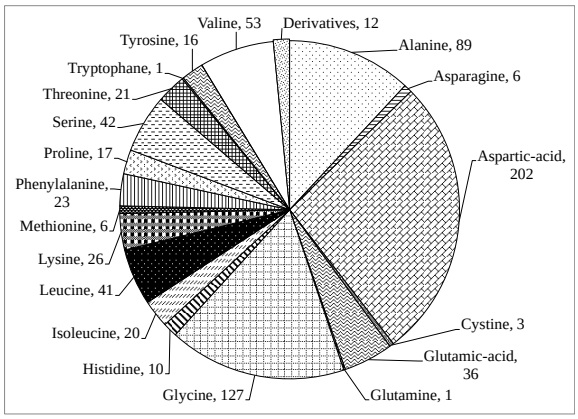

Fig. 5 Amino acid composition (µmol/L) at 280°C

The effect of temperature on the concentration of amino acids was expected to be proportional to its effect on protein extraction considering that amino acids are obtained through hydrolysis of proteins. The results show that this is indeed true. The total concentration of amino acids is highest at 240°C and then decreases with increasing temperature.

In this study, 19 of the 20 primary amino acids were extracted from amaranth stems. Amino acid derivatives such as alpha-aminobutyric-acid (derivative of alanine) and 4-hydroxyproline (derivative of proline) were also present in the samples. Arginine was the only primary amino acid absent although ornithine (a product of the hydrolysis of arginine) was present in small amounts. It has been observed by Murray and co-workers [31] that arginine is converted to ornithine at elevated temperatures which explains the presence of this compound since subcritical hydrolysis extraction of the samples was performed under these conditions.

It was observed that glutamine and asparagine concentrations were much lower than the concentrations of glutamic-acid and aspartic-acid respectively. The reason for this is that asparagine is usually hydrolyzed to aspartic acid and glutamine to glutamic acid at high temperatures [31]. This also explains why these acidic amino acids became more prominent with increasing temperature.

The results show that some of the primary amino acids were hydrolyzed to amino acid derivatives. The percentage of amino acid derivatives increased with increasing temperature. This may be due to the degradation of the amino acids at high temperatures. Under these conditions the C-N bond between the carboxyl and amine group is rapidly hydrolyzed leading to lower amino acid yields and higher degradation [24].

Glycine was observed to be the most prominent amino acid at 240 and 260°C. At 280°C aspartic-acid was more prominent, followed by glycine. As mentioned before, asparagine is hydrolyzed to aspartic-acid at high temperatures which is why it is more prominent at 280°C.

From the results discussed above it is clear that the hydrolysis and degradation of amino acids increases at a higher temperature.

IV. Conclusion

Subcritical water hydrolysis was proven to be an effective technique for the extraction of protein from amaranth stems. High concentrations of proteins were obtained. Amaranth stems were shown to be a valuable source of amino acids with the hydrolyzate containing 19 of the 20 primary amino acids. The highest amount of proteins was extracted at a temperature of 240°C in a 2 g/L HCl solution. The most prominent amino acid obtained from the extracted proteins was glycine. Glycine is the simplest amino acid and is an essential building block in most proteins. It is used in the pharmaceutical industry to produce drugs for treatment of dermatological problems such as eczema and dermatitis.

Acknowledgment

The authors thank the Faculty of Biochemistry at the North West University for their kind assistance with the amino acid analysis and the use of their lab. We extend our acknowledgment to Novozymes for supplying the enzyme solution.

This work is based on the research supported by the National Research Foundation. Any opinion, finding and conclusion or recommendation expressed in this material is that of the author(s) and the NRF does not accept any liability in this regard.

References

[1] B.E. Dale, "'Greening' the chemical industry: research and development priorities for biobased industrial products," *Journal of Chemical Technology and Biotechnology*, vol.78, pp. 1093 – 1103, 2003. http://dx.doi.org/10.1002/jctb.850

[2] C. J. Arntzen, B. E. Dale, N. R. Beachy, R. R. Burgess, P. W. Gallagher, R. W. F. Hardly, D. Johnson *et al*., *Bio-based Industrial Products: Priorities for Research and Commercialization* (Book style)Washington (DC): National Academies Press, 2000, pp. 1.

[3] Y. Sun and J. Cheng, "Hydrolysis of lignocellulosic materials for ethanol production: a review," *Bioresource Technology*, vol. 83, pp. 1 – 11, 2002. http://dx.doi.org/10.1016/S0960-8524(01)00212-7

[4] Y. Lu and R. C. Larock, "Soybean oil-based, aqueous cationic polyurethane dispersions: Synthesis and properties," *Progress in Organic Coatings*, vol. 69, pp. 31– 37, 2010. http://dx.doi.org/10.1016/j.porgcoat.2010.04.024

[5] H. Fukuda, A. Kondo and H. Noda, "Biodiesel Fuel Production by Transesterification of Oils," *Journal of Bioscience and Bioengineering*, vol. 92, no. 5, pp. 405–416, 2001. http://dx.doi.org/10.1016/S1389-1723(01)80288-7

[6] M. Ramezani and S.R. Schmid, "Bio-based lubricants for forming of magnesium," *Journal of Manufacturing Processes*, vol. 19, pp. 112–117, 2015. http://dx.doi.org/10.1016/j.jmapro.2015.06.008

[7] Y.W. Sari, "Biomass and its potential for protein and amino acids; valorizing agricultural by-products," Wageningen University. (Thesis – PhD), pp.14, 2015.

[8] E. Heinzle, A. Biwer and C. Cooney. *Development of Sustainable Bioprocesses*. West Sussex: John Wiley & Sons Ltd, 2, pp.21, 2006 http://dx.doi.org/10.1002/9780470058916.

[9] W. Hettrich and R. Becker, "New isocyanates from amino acids," *Polymer*, vol. 38, no. 10, pp. 2437-2445, 1997. http://dx.doi.org/10.1016/S0032-3861(96)00798-7

[10] E. Scott, F. Peter and J. Sanders, "Biomass in the manufacture of industrial products—the use of proteins and amino acids," *Microbiol Biotechnol*, vol. 75, pp. 751–762, 2007. http://dx.doi.org/10.1007/s00253-007-0932-x

[11] R. Bressani, "Amaranth," *Encyclopedia of Food Sciences and Nutrition*, vol. 2, pp. 166–173, 2003. http://dx.doi.org/10.1016/b0-12-227055-x/00036-5

[12] P. Kraujalis, P.R. Venskutonis, A. Pukalskas and R. Kazernaviciute, "Accelerated solvent extraction of lipids from Amaranthus spp. seeds and characterization of their composition," *Food Science and Technology*, vol. 54, pp. 528–534, 2013. http://dx.doi.org/10.1016/j.lwt.2013.06.014

[13] D. Martínez-Maqueda, B. Hernández-Ledesma, L. Amigo, B. Miralles and J. A. Gómez-Ruiz, "Extraction/Fractionation Techniques for Proteins and Peptides and Protein Digestion". New York: Springer Science & Business Media, 2013. http://dx.doi.org/10.1007/978-1-4614-5626-1_2

[14] A. Segneanu, F. Cziple, P. Vlazan, P. Sfirloaga, I. Grozescu, and V. D Gherman, "Biomass Extraction Methods," Romania: INTECH, 2013. http://dx.doi.org/10.5772/55338

[15] J. S. Hamada, "Characterization of protein fractions of rice bran to devise effective methods of protein solubilisation," *Cereal Chem*, vol. 74, pp. 662–668, 1997. http://dx.doi.org/10.1094/CCHEM.1997.74.5.662

[16] S. S. Natarajan, K. H. B. Lakshman and S. G. M. Garrett, "An efficient extraction method to enhance analysis of low abundant proteins from soybean seed," *Analytic Biochemistry*, vol. 394, no. 1, pp. 259–268, 2009 http://dx.doi.org/10.1016/j.ab.2009.07.048.

[17] D. de Souza, A.F. Sbardelotto, D.R. Ziegler, L.D.F. Marczak, and I.C. Tessaro, "Characterization of rice starch and protein obtained by a fast alkaline extraction method," *Food Chemistry*, vol. 191, pp. 36–44, 2015. http://dx.doi.org/10.1016/j.foodchem.2015.03.032

[18] Y. Yuan and D. Macquarrie, "Microwave assisted extraction of sulfated polysaccharides (fucoidan) from Ascophyllum nodosum and its antioxidant activity," *Carbohydrate Polymers*, vol. 129, pp. 101–107, 2015. http://dx.doi.org/10.1016/j.carbpol.2015.04.057

[19] C. C. Moraes, L. Sala, G. P. Cerveira and S. J. Kalil, "C-phycocyanin extraction from Spirulina platensis wet biomass," *Brazilian Journal of Chemical Engineering*, vol. 28, no. 1, pp. 45–49, 2011. http://dx.doi.org/10.1590/S0104-66322011000100006

[20] P. Kraujalis and P. R. Venskutonis, "Optimisation of supercritical carbon dioxide extraction of amaranth seeds by response surface methodology and characterization of extracts isolated from different plant cultivars," *The Journal of Supercritical Fluids*, vol. 73, pp. 80–86, 2012. http://dx.doi.org/10.1016/j.supflu.2012.11.009

[21] J. Liu, P. Chen, W. Yao, J. Wang, L. Wang, L. Deng, J. He, G. Zhang and J. Lei, "Subcritical water extraction of betulinic acid from birch barks," *Industrial Crops and Products*, vol. 74, pp. 557–565, 2015 http://dx.doi.org/10.1016/j.indcrop.2015.05.064.

[22] X. Liang and Q. Fan, "Application of Sub-Critical Water Extraction in Pharmaceutical Industry," *Journal of Materials Science and Chemical Engineering*, vol. 1, pp. 1–6, 2013. http://dx.doi.org/10.4236/msce.2013.15001

[23] S. Rovio, K. Hartonen, Y. Holm, R. Hiltunen and M. L. Riekkola, "Extraction of Clove Using Pressurized Hot Water," *Flavour and Fragrance Journal*, vol. 14, no. 6, pp. 399–404, 1999. http://dx.doi.org/10.1002/(SICI)1099-1026(199911/12)14:6<399::AID-FFJ851>3.0.CO;2-A

[24] J. L. Garcia-Moscoso, W. Obeid, S. Kumar and P.G. Hatcher, "Flash hydrolysis of microalgae (Scenedesmus sp.) for protein extraction and production of biofuels intermediates," *The Journal of Supercritical Fluids*, vol. 82, no. 1, pp. 183-190, 2013. http://dx.doi.org/10.1016/j.supflu.2013.07.012

[25] I. Sereewatthanawut, S. Prapintip, K. Watchiraruji, M. Goto, M. Sasaki, and A. Shotipruk, "Extraction of protein and amino acids from deoiled

rice bran by subcritical water hydrolysis," *Bioresource Technology*, vol. 99, pp. 555–561, 2007.
http://dx.doi.org/10.1016/j.biortech.2006.12.030

[26] O. A. López-Mejía, A. López-Malo and E. Palou,"Antioxidant capacity of extracts from amaranth (*Amaranthushypochondriacus L.*) seeds or leaves," *Industrial Crops and Products*, vol. 53, pp. 55-59. DATE
http://dx.doi.org/10.1016/j.indcrop.2013.12.017

[27] M. Guo, T. Shi, Y. Duan, J. Zhu, J. Li. And Y. Cao, "Investigation of amino acids in wolfberry fruit (*Lycium barbarum*) by solid-phase extraction and liquid chromatography with precolumn derivatization," *Journal of Food Composition and Analysis*, vol. 42, pp. 84– 90, 2015
http://dx.doi.org/10.1016/j.jfca.2015.03.004.

[28] S. Damrongsakkul, K. Ratanathammapan, K. Komolpis and W. Tanthapanichakoon, "Enzymatic hydrolysis of rawhide using papain and neutrase," *Journal of Industrial and Engineering Chemistry*, vol. 14, pp. 202–206, 2008.
http://dx.doi.org/10.1016/j.jiec.2007.09.010

[29] S. Kumar and R.B. Gupta, "Hydrolysis of microcrystalline cellulose in subcritical and supercritical water in a continuous flow reactor," *Industrial and Engineering Chemistry Research*, vol. 47, pp. 9321–9329, 2008.
http://dx.doi.org/10.1021/ie801102j

[30] M. Fountoulakis and H. Lahm, "Hydrolysis and amino acid composition analysis of proteins," Journal of Chromatography, vol.826, pp. 109–134, 1998.
http://dx.doi.org/10.1016/S0021-9673(98)00721-3

[31] K. Murray, P. S. Rasmussen, J. Neustaedter and J. Murray Luck, "The Hydrolysis of Arginine," The Journal of Biological Chemistry, vol. 240, no. 2, pp. 705–709, 1965.