Jurnal Farmasi dan Ilmu Kefarmasian Indonesia
Pharmacy and Pharmaceutical Sciences Journal
Published by Faculty of Pharmacy Universitas Airlangga
OPEN ACCESS
E-ISSN 2580-8303
P-ISSN 2406-9388

Jurnal Farmasi dan Ilmu Kefarmasian Indonesia Vol. 10 No. 2 August 2023, 210-216
DOI: 10.20473/jfiki.v10i22023.210-216
Available online at https://e-journal.unair.ac.id/JFIKI/

Optimization and Prevalidation of TLC-Densitometry Method for Fucoidan Analysis in *Sargassum sp*. Aqueous Extract

Laili Irfanah[1], Mochammad Yuwono[2*], Riesta Primaharinastiti[2]
[1]Master Program of Pharmaceutical Sciences, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia
[2]Department of Pharmaceutical Sciences, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia

*Corresponding author: yuwono05@yahoo.com

Submitted: 3 April 2023
Revised: 26 June 2023
Accepted: 13 July 2023

Abstract

Background*: Fucoidan is sulfated polysaccharide that has gastroprotector activity, and it is distributed in brown algae cell walls. Currently, there is no method for fucoidan analysis in compendia. Furthermore, analysis of Fucoidan is proven to be challenging due to the lack of chromophores and its high polarity.* ***Objective****: To develop the optimal condition of TLC-Densitometry method for fucoidan analysis in Sargassum sp. aqueous extract and to evaluate the stability of Fucoidan as a preliminary study.* ***Methods****: Chromatography was performed on Silica gel 60F_{254} TLC-plate as a stationary phase. The developed plate was stained with H_2SO_4 10% in absolute ethanol and heated in oven at 105°C for 15 minutes. Optimization is carried out by determining composition of the mobile phase, analytical wavelength, and spotting volume. Stability test of Fucoidan in standard and extract solution at 0, 4, 8, and 24 hours also 0 and 60 minutes after derivatization.* ***Results****: The optimal condition which produces a good separation of Fucoidan was achieved by using n-butanol:methanol: water (10:6:10 v/v/v) as a mobile phase, 400 nm as an analytical wavelength, and 1 µl as a spotting volume. Fucoidan was stable after storage until 24 hours. The stained spots were stable until 60 minutes after derivatization.* ***Conclusion****: Optimal condition of the TLC-Densitometry method for Fucoidan analysis was selective and can be applied to stability tests in preliminary study. Fucoidan was stable in standard solution and extracted solution until 24 hours after storage at 4°C, and the stained spots were stable until 60 minutes after derivatization.*

Keywords*: fucoidan, prevalidation, Sargassum sp., TLC-densitometry*

How to cite this article:
Irfanah, L., Yuwono, M. & Primaharinastiti, R. (2023). Optimization and Prevalidation of TLC-Densitometry Method for Fucoidan Analysis in *Sargassum sp*. Aqueous Extract. *Jurnal Farmasi dan Ilmu Kefarmasian Indonesia*, 10(2), 210-216. http://doi.org/10.20473/jfiki.v10i22023.210-216

INTRODUCTION

As a maritime country, Indonesia has high marine potential. One of these is the existence of *Sargassum sp.*, which consists of approximately 400 species spread across the Java Sea to the Banda Sea. *Sargassum sp.* contain bioactive compounds that can be utilized in the health sector (Rohim *et al*., 2019). Fucoidan is a bioactive compound in *Sargassaum sp*. that has gastroprotective activity (Hu *et al*., 2020). Fucoidan (Table 1) is a type of sulfate polysaccharide found in the cell wall of *Sargassum sp.*, which contains fucose as its main component (Li *et al*., 2017).

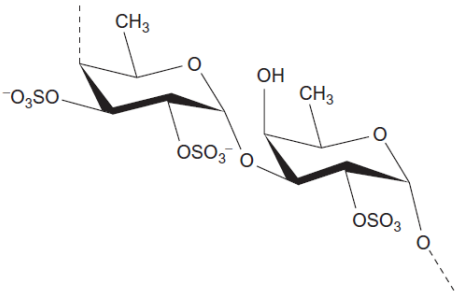

Figure 1. Chemical Structure of Fucoidan

Sargassum sp. extract is used as an industrial raw material and source of fucoidan. Therefore, finding a reliable, fast, and inexpensive method for fucoidan analysis is very important. However, there is no analytical method for fucoidan analysis in the compendia. Therefore, an analytical method for fucoidan analysis must be developed. Several analytical methods for fucoidan analysis have been developed, including fluorometric assays and UV spectrophotometry (Venkatesan *et al*., 2018; Yamazaki *et al*., 2016). However, because herbal extracts are composed of several substances, they are not likely to be simple to apply, and the process requires the isolation of fucoidan from the other components. Chromatography is a method used to separate multiple compounds. High-performance liquid chromatography (HPLC) methods for fucoidan analysis use photodiode array (PDA), refractive index (RI), and mass spectrometry (MS/MS) detectors (Isnansetyo *et al*., 2017; Zhao *et al*., 2021; Zhu *et al*., 2018).

In addition to HPLC, thin-layer chromatography (TLC) can also be used for fucoidan analysis. The TLC method was used to separate compounds, such as fucoidan, from the crude extract of *Sargassum turbinarioides* and *Sargassum ilicifolium* for screening crude extracts (Artemisia *et al*., 2019). In that study, a densitometer was not used, whereas the detector contained in the densitometer allowed the TLC method to achieve accurate qualitative and quantitative results (Ekasari *et al*., 2020). Therefore, a TLC densitometry method for fucoidan analysis was developed. TLC densitometry is a relatively simpler and less time-consuming chromatography method than HPLC (Ahmad *et al*., 2020; Ekasari *et al*., 2020; Satpathy *et al*., 2017; Sherma & Rabel, 2018). Fucoidan analysis with TLC densitometry was challenging because of the lack of a chromophore group and its high polarity. In this study, the development of a TLC-densitometry method for fucoidan analysis was carried out at an optimization stage to determine the optimal condition of the TLC-densitometry method for fucoidan analysis in *Sargassum sp.* aqueous extract and to evaluate the stability of fucoidan as a preliminary study.

MATERIALS AND METHODS

Materials

Fucoidan standard (Sigma Aldrich) and *Sargassum sp.* extract powder were obtained from the pharmaceutical industry in Indonesia (PT. Natura Laboratoria Prima), methanol (Merck), and water (PT. Ikapharmindo Putramas), n-butanol (Merck), n-hexane (Merck), acetone (Merck), ethyl acetate (Merck), isopropanol (Merck), acetonitrile (Merck), ethanol (Merck), sulfuric acid (Merck), and silica gel $60F_{254}$ TLC) aluminum plates (20 × 20 cm (Merck).

Tools

Analytical balance (Mettler Toledo), Twin Trough Chamber 20 × 20 cm (Camag), Linomat 5 (Camag), TLC Scanner 4 with a UV detector (Camag), VisionCATS software (Camag), oven (Medcenter), and reagent sprayer.

Method

Preparation of standard solution

A standard solution (2000 µg/ml) was prepared by dissolving 10 mg of the fucoidan standard in 5 ml of water: methanol 1:9 (v/v). The mixture was then vortexed until homogenous.

Preparation of sample solution

Sargassum sp. extract powder (10 mg) was dissolved in 5 ml of water: methanol 1:9 (v/v). The mixture was then vortexed until homogenous.

Preparation of mobile phase

The mobile phase was freshly mixed with various compositions for the optimization stage, as shown in Table 1. The development chamber was then left to saturate with mobile phase vapor for 2 h before each eluation.

P-ISSN: 2406-9388
E-ISSN: 2580-8303

Table 2. Optimization parameters

No.	Optimization	Parameter	Criteria
1.	Composition of the mobile phase		
	(1) n-hexane : acetone (4:6, v/v)		
	(2) Acetone : water (7:3, v/v)	Rf	0.2-0.8
	(3) Isopropanol : ethyl acetate : water (7:2:1, v/v/v)	Rs	≥ 1.5-2.0
	(4) Acetonitrile : n-butanol : water (6:3:1, v/v/v)		
	(5) n-butanol : methanol : water (10:6:10, v/v/v)		
2.	Analytical wavelength		
	fucoidan spot was tested for its UV spectrum profile at wavelengths of 200-700 nm	Maximum wavelength	Maximum wavelength (based on UV spectrum)
3.	Spotting volume		
	(1) 1 µl	Area and Peak Shape (Symmetry factor (As))	Largest area with symmetry peak shape (As : 0.9-1.2)
	(2) 2 µl		
	(3) 3 µl		

Table 1. Variation of mobile phase composition

No.	Mobile phase composition
1.	n-hexane : acetone (4:6, v/v)
2.	Acetone : water (7:3, v/v)
3.	Isopropanol : ethyl acetate : water (7:2:1, v/v/v)
4.	Acetonitrile : n-butanol : water (6:3:1, v/v/v)
5.	n-butanol : methanol : water (10:6:10, v/v/v)

Optimization of analytical conditions

Optimization was performed by determining the composition of the mobile phase, analytical wavelength, and spotting volume. The parameters observed in this optimization stage are listed in Table 2. The chamber was saturated with the mobile phase. Standard and sample solutions were added to the plates. The plate was then developed in a saturated chamber until a migration distance of 70 mm from the origin was reached. After development, the plate was dried with a hair dryer for 20 min at room temperature and then dried in an oven for 15 min at 40°C. To visualize the spots, the plate was sprayed with H_2SO_4 10% in absolute ethanol and then heated in an oven at 105°C for 15 min. The absorbance of fucoidan from yellow to dark spots was measured using a densitometer.

A mobile phase that could separate fucoidan from other compounds with a resolution (Rs) value ≥ 1.5-2.0 and had a retardation factor (Rf) range of 0.2-0.8 was chosen. The analytical wavelength and spotting volume that produced the largest area and sharper peaks were selected. The symmetry factor (As) is a parameter that can characterize the shape of the peaks. The optimal values for the symmetry factor of the peaks were 0.9–1.2 (Czyrski & Sznura, 2019).

Stability test for standard and extract solution

The stability test is a pre-validation stage that aims to determine the stability of fucoidan in standard and extract solutions. Standard and extract solutions were spotted, developed, and analyzed at 0, 4, 8, and 24 h after storage at 4°C. The stability of the resulting spots after derivatization was also tested 0 and 60 min after derivatization.

RESULTS AND DISCUSSION

Optimization was performed by determining the composition of the mobile phase, analytical wavelength, and spotting volume. In this optimization stage H_2SO_4 10% in absolute ethanol was used as the visualizer reagent because of the lack of a chromophore group in fucoidan. Mobile phase optimization is a crucial step in TLC method development, because it ultimately affects the quality of separation (Chaudhari & Shirkhedkar, 2020). The results of the mobile phase optimization are listed in Table 3. Based on mobile phase optimization, only the n-butanol: methanol: water (10:6:10, v/v/v) mobile phase could separate fucoidan from other compounds. The resolution (Rs) value and the retardation factor (Rf) value of the fucoidan peak, were 0.58 and 3.60, respectively. It means that this mobile phase can produce an optimal separation of fucoidan spot with another compound (Rs ≥ 1.5-2.0), and the Rf value met the requirement range of 0.2-0.8 (AOAC International, 2019; Indrayanto *et al*., 2009; Yuwono & Indrayanto, 2005). Meanwhile, the results of fucoidan analysis using various other mobile phases showed that fucoidan was still at the starting point of the spotting area, so the resulting Rf value of fucoidan spots was around 0 (out of the requirement range), and the separation process could not occur. This could be due to

P-ISSN: 2406-9388
E-ISSN: 2580-8303

the high polarity of fucoidan, and the polarity of the stationary phase is also polar, so the fucoidan is strongly bonded with the stationary phase (Zayed *et al*., 2020). In this case, a mobile phase with high polarity is required, resulting in the optimal Rf value for fucoidan. Therefore, n-butanol: methanol: water (10:6:10, v/v/v) was selected as the mobile phase because it can migrate the fucoidan spot until it meets the requirement for the Rf value and can optimally separate fucoidan with good Rs values. Different compositions of the mobile phase also differ in polarity, where the separation process depends on the polarity of the mobile phase, which affects the Rf and Rs values.

Table 3. The results of fucoidan analysis using a variation of mobile phase composition

Mobile Phase	Rf	Rs
1	0.01	25.21
2	0.01	3.00
3	0.01	5.85
4	0.01	8.24
5	0.58	3.15

Subsequently, the fucoidan spot was tested for its UV spectral profile. Spectral measurements were performed at wavelengths of 200-700 nm. The Fucoidan spectrum is shown in Figure 2. The fucoidan spectrum shows that the maximum wavelength of fucoidan is 400 nm, which provides the maximum absorption for fucoidan. Therefore, 400 nm was selected as the analytical wavelength of fucoidan. TLC densitograms of fucoidan in the standard and extract solutions at a wavelength of 400 nm are shown in Figure 3.

Spotting volume optimization was conducted by spotting fucoidan standard with various spotting volumes such as 1 µL, 2 µL, and 4 µL on the stationary phase, and then analyzed according to a previous procedure, including scanning with a densitometer at 400 nm wavelength. The observed response areas and peak shapes of fucoidan with various spotting volumes are shown in Table 4. Based on the results in Table 4, the largest response area was produced by 3 µL, but the peak shape was asymmetric (fronting). A spotting volume of 2 µL also produces asymmetry (fronting). A spotting volume of 1 µL was chosen for each analysis because it can produce a symmetrical peak shape, although with the lowest response area. The amount of sample to be applied in a spot is sometimes difficult to determine because it depends on several variables, such as the sample matrix itself, sorbent thickness, and sample solvent. If the applied sample is overloaded, poor chromatographic separation on thin layers will occur (Wall, 2005). This is the reason why the spotting volume needs to be optimized.

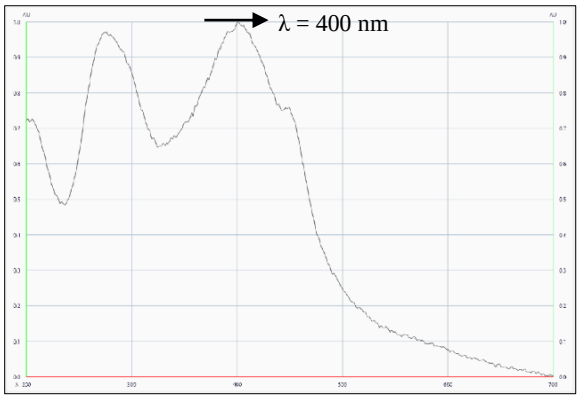

Figure 2. The fucoidan spectrum at a wavelength 200-700 nm

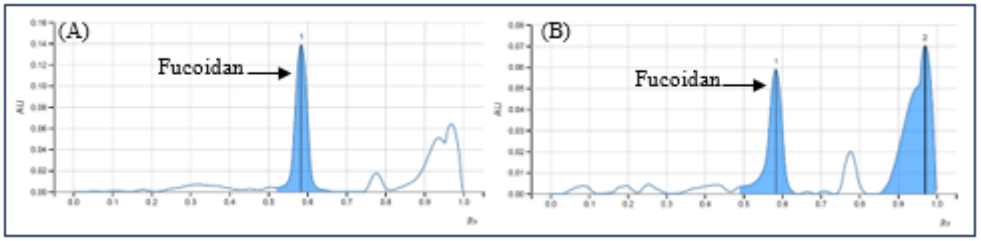

Figure 3. TLC densitograms of Fucoidan in standard (A) and extract (B) solution at a wavelength of 400 nm

P-ISSN: 2406-9388
E-ISSN: 2580-8303

Table 4. The observed response area and peak shape of Fucoidan with various spotting volumes

Concentration	Spotting Volume	Area	As	Peak Shape
2 µg/µl	1 µl	0.00446	0.90	Symmetry
2 µg/µl	2 µl	0.00687	0.71	Asymmetry
2 µg/µl	3 µl	0.00904	0.77	Asymmetry

Table 5. Stability test results for fucoidan standard and extract solution

Spotting time (hours)	0 min after derivatization		60 min after derivatization	
	Average Area of Standard Solution	Average Area of Extract Solution	Average Area of Standard Solution	Average Area of Extract Solution
0	0.00676	0.00390	0.00637	0.00374
4	0.00572	0.00317	0.00584	0.00327
8	0.00719	0.00427	0.00768	0.00448
24	0.00649	0.00497	0.00664	0.00535

The results of the stability test of fucoidan in a preliminary study are shown in Table 5. One-way ANOVA statistical analysis was performed with a 95% confidence interval ($\alpha = 0.05$) on the fucoidan area from each spotting time. The one-way ANOVA results showed a significance value of 0.068 for the standard solution and 0.050 for the extract solution. The significance value was more than α (0.05). This indicates that there was no significant difference in the fucoidan area obtained from each spotting time in the standard and extract solutions. It can be concluded that fucoidan is stable until 24 h of storage at 4°C. A paired t-test statistical analysis was carried out with a 95% confidence interval ($\alpha = 0.05$) on the results of the fucoidan standard and extract solution area at 0 and 60 min after derivatization. The paired t-test statistical analysis results showed that the significance values of the standard solution and extract solution areas between 0 and 60 min after derivatization were 0.488 and 0.253, respectively. This indicates that there was no significant difference between the areas observed at 0 and 60 min after derivatization. Therefore, it can be assumed that the derivatized stain is stable up to 60 min after derivatization.

CONCLUSION

The optimal conditions for Fucoidan analysis were n-butanol: methanol: water (10:6:10, v/v/v) as the mobile phase, 400 nm as the analytical wavelength, and 1 µL as the spotting volume. This method was selective and can be applied to stability tests in a preliminary study. Fucoidan was stable in the standard solution and extracted solution until 24 h after storage at 4°C, and the stability of stained spots was stable until 60 min after derivatization. This optimal condition can be validated in future research.

ACKNOWLEDGMENT

All authors are grateful to all parties who contributed to the creation.

AUTHOR CONTRIBUTIONS

Conceptualization, L. I., M. Y., R. P.; Methodology, L. I., M. Y., R. P.; Software, L. I.; Validation, L. I.; Formal Analysis, L. I.; Investigation, L. I.; Resources, L. I., M. Y., R. P.; Data Curation, L. I., M. Y., R. P.; Writing - Original Draft, L. I., M. Y., R. P.; Writing - Review & Editing, L. I., M. Y., R. P.; Visualization, L. I., M. Y., R. P.; Supervision, L. I., M. Y., R. P.; Project Administration, L. I., M. Y., R. P.; Funding Acquisition, L. I., M. Y., R. P.

FUNDING STATEMENT

This research did not receive any specific grant from funding agencies in the public, commercial, or not for profit sectors.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

REFERENCES

Ahmad, A., Amir, M., Alshadidi, A. A., Delwar, M., Haq, A. & Kazi, M. (2020). Central composite Design Expert-Supported Development and Validation of HPTLC Method: Relevance in Quantitative Evaluation of Protopine in *Fumaria indica*. *Saudi Pharmaceutical Journal*; *28*; 487–494. doi: 10.1016/j.jsps.2020.02.011.

AOAC International. (2019). Guideline for Dietary Supplements and Botanical (Appendix K). Rockville: AOAC International.

Artemisia, R., Nugroho, A. K., Setyowati, E. P. & Martien, R. (2019). The Properties of Brown Marine Algae Sargassum turbinarioides and

Sargassum ilicifolium Collected From Yogyakarta, Indonesia. *Indonesian Journal of Pharmacy*; *30*; 43-51. doi: 10.14499/indonesianjpharm30iss1pp43.

Chaudhari, S. R. & Shirkhedkar, A. A. (2020). Application of Plackett-Burman and Central Composite Designs for Screening and Optimization of Factor Influencing the Chromatographic Conditions of HPTLC Method for Quantification of Efonidipine Hydrochloride. *Journal of Analytical Science and Technology*; *11*; 1-13. doi: 10.1186/s40543-020-00246-2.

Czyrski, A. & Sznura, J. (2019). The Application of Box-Behnken-Design in the Optimization of HPLC Separation of Fluoroquinolones. *Scientific Reports*; *9*; 1–10. doi: 10.1038/s41598-019-55761-z.

Ekasari, W., Widiyastuti, Y., Subositi, D., Hamsidi, R., Widyawaruyanti, A., Basuki, S. & Setyawan, D. (2020). Determination of Cassiarin A Level of *Cassia siamea* Leaf Obtained from Various Regions in Indonesia Using the TLC-Densitometry Method. *Scientific World Journal*; *2020*; 1-7. doi: 10.1155/2020/7367836.

Hu, Y., Ren, D., Song, Y., Wu, L., He, Y., Peng, Y., Zhou, H., Liu, S., Cong, H., Zhang, Z., & Wang, Q. (2020). Gastric Protective Activities of Fucoidan from brown Alga Kjellmaniella Crassifolia Through the NF-κB Signaling Pathway. *International Journal of Biological Macromolecules*; *149*; 893–900. doi: 10.1016/j.ijbiomac.2020.01.186.

Indrayanto, G., Yuwono, M., & Suciati. (2009). TLC: Validation of Analyses. In *Encyclopedia of Chromatography*. Milton Park: Taylor & Francis.

Isnansetyo, A., Lutfia, F. N. L., Nursid, M., Trijoko, T., & Susidarti, R. A. (2017). Cytotoxicity of Fucoidan from three Tropical Brown Algae Against Breast and Colon Cancer Cell Lines. *Pharmacognosy Journal*; *9*; 14–20. doi: 10.5530/pj.2017.1.3.

Li, G. Y., Luo, Z. C., Yuan, F., & Yu, X. (2017). Combined Process of High-Pressure Homogenization and Hydrothermal Extraction for the Extraction of Fucoidan with Good Antioxidant Properties from *Nemacystus decipiens*. *Food and Bioproducts Processing*; *106*; 35–42. doi: 10.1016/j.fbp.2017.08.002.

Rohim, A., Yunianta, & Estiasih, T. (2019). Senyawa-Senyawa Bioaktif Pada Rumput Laut Cokelat Sargassum Sp. : Ulasan Ilmiah. *Jurnal Teknologi Pertanian*; *20*; 115–126. doi: 10.21776/ub.jtp.2019.020.02.5.

Satpathy, S., Patra, A., Hussain, M. D., & Ahirwar, B. (2017). Simultaneous Estimation of Genistein and Daidzein in Pueraria Tuberosa (Willd.) DC by Validated High Performance Thin Layer Chromatography (HPTLC) Densitometry Method. *Journal of Liquid Chromatography & Related Technologies*; *40*. doi: 10.1080/10826076.2017.1329743.

Sherma, J., & Rabel, F. (2018). A Review of Thin Layer Chromatography Methods for Determination of Authenticity of Foods and Dietary Supplements. *Journal of Liquid Chromatography and Related Technologies*; *41*; 645–657. doi: 10.1080/10826076.2018.1505637.

Venkatesan, J., Singh, S. K., Anil, S., Kim, S. K., & Shim, M. S. (2018). Preparation, Characterization and Biological Applications of Biosynthesized Silver Nanoparticles with Chitosan-Fucoidan Coating. *Molecules*; *23*; 1-12. doi: 10.3390/molecules23061429.

Wall, P. E. (2005). RSC Chromatography Monographs: Thin-layer Chromatography. London: The Royal Society of Chemistry.

Yamazaki, Y., Nakamura, Y., & Nakamura, T. (2016). A Fluorometric Assay for Quantification of Fucoidan, A Sulfated Polysaccharide from Brown Algae. *Plant Biotechnology*; *33*; 117–121. doi: 10.5511/plantbiotechnology.16.0217c

Yuwono, M., & Indrayanto, G. (2005). Validation of Chromatographic Methods of Analysis. *Profiles of Drug Substances, Excipients and Related Methodology*; *32*; 241–260. doi: 10.1016/S0099-5428(05)32009-0.

Zayed, A., El-Aasr, M., Ibrahim, A. R. S., & Ulber, R. (2020). Fucoidan Characterization: Determination of Purity and Physicochemical and Chemical Properties. *Marine Drugs*; *18*; 1–31. doi: 10.3390/md18110571.

Zhao, M., Garcia-Vaquero, M., Przyborska, J., Sivagnanam, S. P., & Tiwari, B. (2021). The Development of Analytical Methods for the Purity Determination of Fucoidan Extracted from Brown Seaweed Species. *International Journal of Biological Macromolecules*; *173*; 90–98. doi: 10.1016/j.ijbiomac.2021.01.083.

P-ISSN: 2406-9388
E-ISSN: 2580-8303

Zhu, Z., Zhu, B., Ai, C., Lu, J., Wu, S., Liu, Y., Wang, L., Yang, J., Song, S., & Liu, X. (2018). Development and Application of a HPLC-MS/MS Method for Quantitation of Fucosylated Chondroitin Sulfate and Fucoidan in Sea Cucumbers. *Carbohydrate Research*; *466*; 11–17. doi: 10.1016/j.carres.2018.07.001.

P-ISSN: 2406-9388
E-ISSN: 2580-8303