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Study of phospholipid molecular species of the green seaweed *Halimeda incrassata* Lamx. from Truong Sa islands, Vietnam

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Abstract
In this report, by a high performance liquid chromatography (HPLC) - high resolution mass spectrometry (HRMS) method, 7 molecular species of phospholipid in the polar lipid class were identified including phosphatidylinositol (PI) and phosphatidylglycerol (PG). The PI 32:0 (16:0/16:0) and PG 34:3 (16:1/18:2) molecular species have the highest content. PI molecular species are mainly formed by saturated fatty acids (16:0, 18:0) and only one polyunsaturated fatty acids C20:4, while PG species are formed by unsaturated fatty acids (16:1, 18:2 and 18:3) and only one saturated fatty acid C16:0.

Keywords: Phospholipid, molecular species, calcified seaweeds, *Halimeda incrassata*, Truong Sa islands.

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INTRODUCTION

The seaweed genus *Halimeda*, belonging to the Halimedaceae family, the Chlorophyta phylum, widely spread over the world especially near the outlying islands in the tropical sea. This species in combination with corals is regarded as a structural component of reefs and also protects seabed from disasters and coastal currents [1]. In the marine environment, the spread of seaweed across seabed is not only an aesthetic scene but also a place for marine animals to spawn and prevent their offspring from danger. In Vietnam, *Halimeda incrassate* Lamx. is mainly distributed in Truong Sa archipelago (Khanh Hoa) [2]. This is a rare marine plant that $CaCO_3$ is synthesized in the tissues (around 35–45%) at a speed equivalent to those of the reefs, thus it is named a calcified seaweed [3]. *Halimeda incrassata* is a potential resource of compounds with various bioactivities such as antibacterial, anti-inflammation,... [4, 5]. Along with natural products from marine organism, lipid is increasingly recognized because of its positive effects on bones, cardiovascular and nervous systems. The effects of lipid are mainly related to the biological and pharmacological roles of polyunsaturated fatty acids (PUFAs) [6–8].

In this report, we aim to report the composition and the content of fatty acids, lipid classes and especially phospholipid molecular species of the green algae *Halimeda incrassata* Lamx. collected from Truong Sa islands. As well known, Truong Sa is a place with the harsh weather conditions, difficulties in commuting and collecting samples. Thus, this publication would greatly contribute practical effects in the novel knowledge of seaweed with large yield in the far islands.

METHODS

Sample collection

The *Halimeda incrassata* Lamx. sample was collected in 7/2017 by DSc. Dam Duc Tien from Truong Sa archipelago, Khanh Hoa, Viet Nam. The sample collecting process has been performed following the Provisional Regulations for Field Survey issued by the State Committee for Science and Technology Vietnam in 1980 [9]. *H. incrassata* sample was then treated and soaked in formaldehyde 5% before identifying the Latin name. The specimen of sample was stored at the Institute of Marine Environment and Resources and the Institute of Natural Products Chemistry (VAST).

Total lipid extraction

Total lipid was extracted with a solvent system of chloroform and methanol (1:2) [10] in the combination with sonication. 100 g of seaweed was extracted two times by the solvent system of 300 ml $CHCl_3$:CH_3OH and 100 ml $CHCl_3$ under ultrasound condition for 180 minutes. The extracted mixture was obtained by filtering, and shaken after adding 80 ml H_2O. The lower layer was collected using funnel. The total lipid was obtained using vacuum rotary evaporator Eyela N1300, then stored at (-)20^oC prior to analysis.

Analysis of molecular species of phospholipid

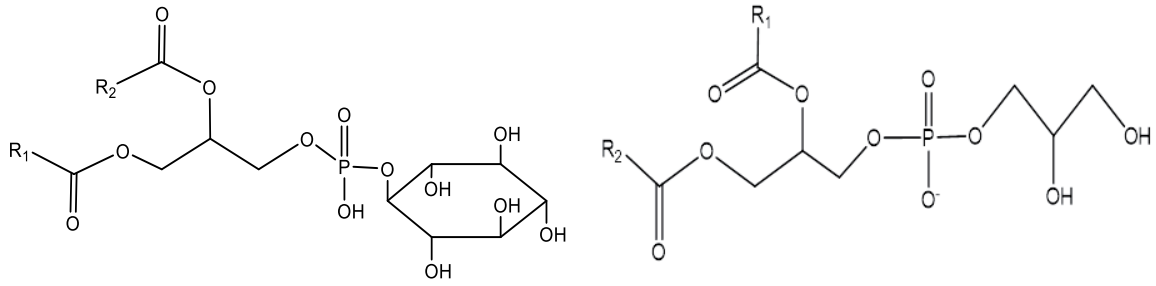
The total lipid was dissolved in MeOH and processed in HPLC-HRMS Shimadzu to recognize molecular species of the phospholipid subclass [11]. The chromatography system contains 2 high-pressure pumps, CTO-20A oven, Shim-Pack diol column (50 mm × 4.6 mm ID, 5 μm, Shimadzu, Kyoto, Japan), using two solvent systems of A hexane:2-propanol:CH_3COOH:$N(CH_3)_3$ 82:17:1:0.08 (v/v) and B (propanol-2): H_2O:CH_3COOH:$N(CH_3)_3$ 85:14:1:0.08 (v/v). Phospholipid molecular species are identified by the combination IT and TOF techniques in the Shimadzu LCMS-IT-TOF device (Kyoto, Japan). The machine could be operated in both negative and positive ion modes in each analysis under electrospray ionisation (ESI) conditions. The quantification of the phospholipid molecular species was calculated according to the area per substance peak obtained on the positive or negative ion spectrums.

RESULT AND DISCUSSION

Identifying molecular species of phospholipid

In the total lipid of the green algae *H. incrassata* sample, polar lipid class (Pol) has accounted for 32.3% of the total lipid. Using

HRMS technique, molecular species in phospholipid (PL) subclass have been identified and classified into 2 groups of phosphatidylinositol (PI) and phosphatidylglycerol (PG) (figure 1). Molecular and ion forms of each PL have been analyzed and determined on the mass spectrum [11].



PI PG

Figure 1. Formula of PI and PG molecular species

Identifying molecular species of phosphatidylinositol (PI)

In the PI group of the green algae *H. incrassta*, 3 molecular species have been identified, in which alkenyl acyl glycerophosphoinositol has not appeared, all of molecular species are diacylglycerophosphoinositol (table 1).

Table 1. Composition of PI's molecular species

Phospholipid (C:N)*	Diacyl	$[M-H]^-$ *m/z*	Molecular formula	Area	% in PI
PI 32:0	16:0/16:0	809.5076	$C_{41}H_{79}O_{13}P$	24745446	52.03
PI 34:0	16:0/18:0	837.5406	$C_{43}H_{83}O_{13}P$	8401740	17.66
PI 36:4	16:0/20:4	857.4986	$C_{45}H_{79}O_{13}P$	27318082	30.31
			∑	47563676	100.00

Note: *: The number of carbon atoms (C) and number of double bonds (N) in the acyl chain.

For example, PI 32:0 has the highest percentage of 52.03% (table 1, figure 2).

On the MS^- spectrum, the ion peak $[M-H]^-$ has the strongest signal at *m/z* 809.5076 $[C_{41}H_{78}O_{13}P]^-$ corresponding to molecular formula $C_{41}H_{79}O_{13}P$ (*m/z* 810.5104). This negative ion was chosen to analyze on the MS^{2-}. MS^{2-} spectrum of PI 32:0 also has the *m/z* signals corresponding to a half-molecular ion containing inositol, acyl group and anion carboxylate of the fatty acid disappearing simultaneously. The signal at *m/z* 553.2717 corresponds to the lost neutral fragment at *m/z* 256.2359 ($C_{16}H_{32}O_2$) (fatty acid 16:0). The signal at *m/z* 391.224 corresponds to ion $[C_{41}H_{78}O_{13}P]^-$ at *m/z* 809.5076 losing both the fragment *m/z* 256.2307 ($C_{16}H_{32}O_2$) (fatty acid 16:0) and inositol *m/z* 162.0528 ($C_6H_{10}O_5$). Besides, the signal at *m/z* 255.2285 on the MS^{2-} is the mass of anion $[C_{16}H_{31}O_2]^-$ (fatty acid 16:0). In conclusion, the spectrum data have shown that the considered molecular species is diacylglycerophosphoinositol PI 16:0/16:0.

According to table 1, the PI's molecular species with the content ranked second is PI 36:4 (30.31%).

On the MS^- spectrum of PI, the signal $[M-H]^-$ at *m/z* 857.4986 $[C_{45}H_{78}O_{13}P]^-$ has been identified with formula of $C_{45}H_{79}O_{13}P$ (*m/z* 858.5161). On the MS^{2-} spectrum of ion $[M-H]^-$ (figure 3c), the ion fragment at the signal at *m/z* 601.2548 is similar to the lost fragment of fatty acid 16:0 ($C_{16}H_{32}O_2$) at *m/z* 256.2438. Additionally, the signal at *m/z* 601.2548 corresponds to ions of glycerophosphate $[C_3H_6O_5P]^-$ at *m/z* 152.9980, inositol ($C_6H_{10}O_5$) at *m/z* 162.0528 and ketene of fatty acid 20:4 ($C_{20}H_{30}O$) at *m/z* 286.2040 (a fatty acid lost 1

H_2O). Hence, PI 36:4 is characteristic of diacylglycerophosphoinositol PI 16:0/20:4. Similarly, the other molecular species of PI 34:0 (PI 16:0/18:0) has been identified in table 1.

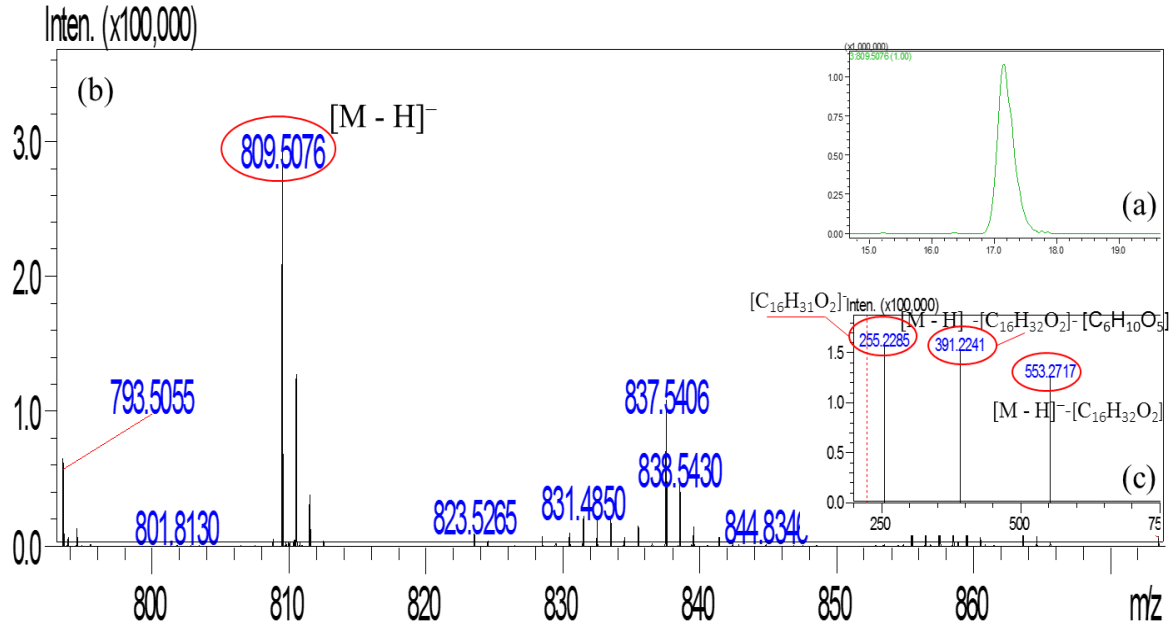


Figure 2. HPLC-HRMS and fragmentation of PI 32:0 $[C_{41}H_{78}O_{13}P]^-$: a- HPLC chromatogram of $[C_{41}H_{78}O_{13}P]^-$; b- Mass spectrometry (MS^-) of $[C_{41}H_{78}O_{13}P]^-$; c- Mass spectrometry (MS^{2-}) of signal at *m/z* 809.5076

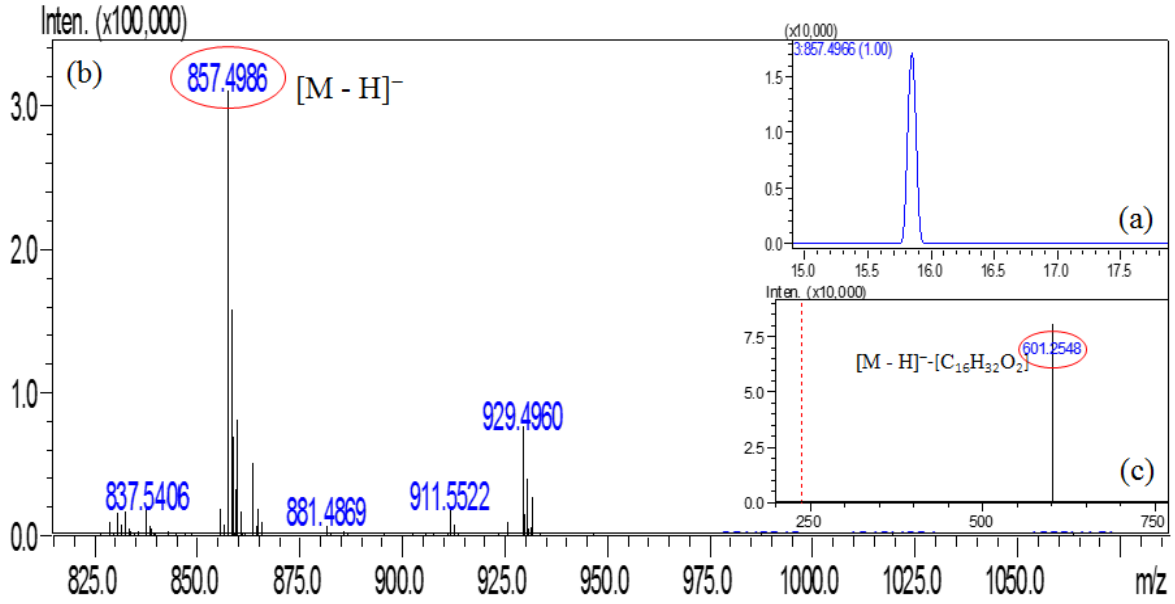


Figure 3. HPLC-HRMS and the fragmentation of PI 36:4 $[C_{45}H_{78}O_{13}P]$: a- HPLC chromatogram of $[C_{45}H_{78}O_{13}P]^-$; b- Mass spectrometry (MS^-) of $[C_{45}H_{78}O_{13}P]^-$; c- Mass spectrometry (MS^{2-}) of signal at *m/z* 857.4986

Identifying molecular species of phosphatidylglycerol (PG)

In the PG group of green algae *H. incrassate*, 4 molecular species with exact formula have been identified (table 2).

Data from table 2 presented that PG 34:3 has the highest content of 61.02% (figure 4). On the negative ion MS^- spectrum of PG, the strongest signal of the negative ion $[M-H]^-$ at *m/z* 743.4772 $[C_{40}H_{72}O_{10}P]^-$ has been recorded. Thus, the formula has been considered $C_{40}H_{73}O_{10}P$ (*m/z* 744.4758).

This anion has been chosen to analyze on the MS^{2-} spectrum. On the negative ion MS^{2-} spectrum of $[M-H]^-$ (figure 4c), the observed ion fragment at *m/z* 507.2671 corresponded to the ion $[C_{40}H_{72}O_{10}P]^-$ losing a ketene of the fatty acid 16:1 at *m/z* 236.2101 ($C_{16}H_{28}O$) (a fatty acid removed 1 H_2O). The signal at *m/z* 415.2241 corresponds to the disappearance of both ketene of the 16:1 fatty acid *m/z* 236.2101 and glycerol ($C_3H_8O_3$) *m/z* 92.0430 from considered ion $[C_{40}H_{72}O_{10}P]^-$. The ion fragment at *m/z* 279.2294 is the segment of anion $[C_{18}H_{31}O_2]^-$ (fatty acid 18:2). Hence, PG 34:3 has been assumed as the character of diacylglycerolphosphoglyerol, PG 16:1/18:2.

Table 2. Composition of PG molecular species

Phospholipid (C:N)*	Diacyl	$[M-H]^-$ *m/z*	Molecular formula	Area	% in PG
PG 32:2	16:1/16:1	717.4629	$C_{38}H_{71}O_{10}P$	10909490	7.81
PG 34:4	16:1/18:3	741.4642	$C_{40}H_{71}O_{10}P$	9443406	6.76
PG 34:3	16:1/18:2	743.4772	$C_{40}H_{73}O_{10}P$	85255153	61.02
PG 34:2	16:0/18:2	745.4902	$C_{40}H_{75}O_{10}P$	34097828	24.41
			∑	139705877	100.00

Note: *: The number of carbon atoms (C) and number of double bonds (N) in the acyl chain.

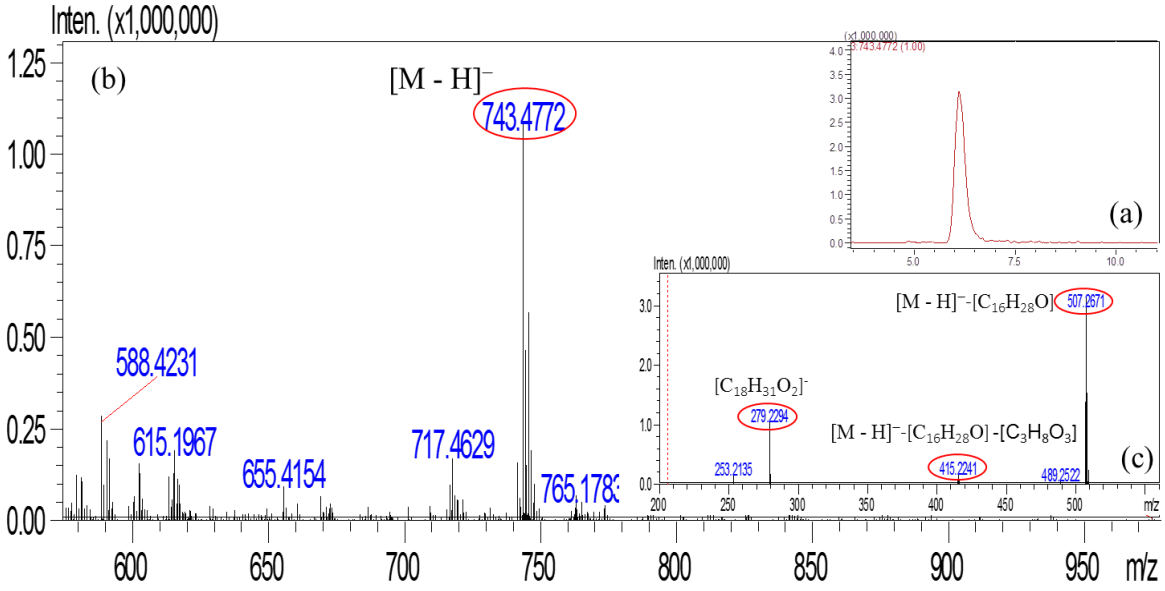


Figure 4. HPLC-HRMS and the fragmentation of PG 34:3 $[C_{40}H_{72}O_{10}P]^-$: a- HPLC chromatogram of $[C_{40}H_{72}O_{10}P]^-$; b- Mass spectrometry (MS^-) of $[C_{40}H_{72}O_{10}P]^-$; c- Mass spectrometry (MS^{2-}) of signal at *m/z* 743.4772

Moreover, table 2 also shows that the lowest content of PG (6.76%) is PG 34:4 (figure 5). The negative ion MS^- spectrum of PG shows the signal at *m/z* 741.4642 $[C_{40}H_{70}O_{10}P]^-$. Thus, the formula is $C_{40}H_{71}O_{10}P$ with *m/z* 742.4578.

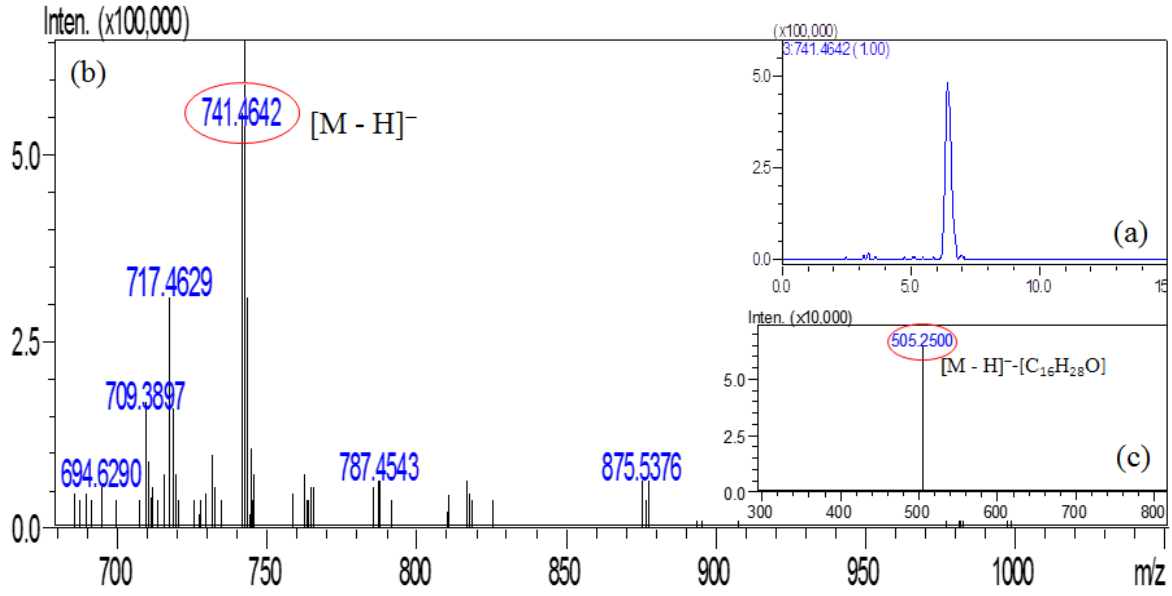


Figure 5. HPLC-HRMS and the fragmentation of PG 34:4 $[C_{40}H_{70}O_{10}P]^-$: a- HPLC chromatogram of $[C_{40}H_{70}O_{10}P]^-$; b- Mass spectrometry (MS^-) of $[C_{40}H_{70}O_{10}P]^-$; c- Mass spectrometry (MS^{2-}) of signal at *m/z* 741.4642

On the MS^{2-}spectrum of $[M-H]^-$ (figure 5c), the ion fragment at *m/z* 505.2500 corresponds to the lost ketene of fatty acid 16:1 at *m/z* 236.2142 ($C_{16}H_{28}O$). On the other hand, the signal at *m/z* 505.2500 is created from the glycerophosphate segment at *m/z* 152.998 $[C_3H_6O_5P]^-$, glycerol molecule at *m/z* 92.0430 ($C_3H_8O_3$) and a ketene of the fatty acid 18:3 ($C_{18}H_{28}O$) (a fatty acid 18:3 lost 1 H_2O) *m/z* 260.2090. Hence, PG 34:4 is regarded as the character of diacylglycerolphosphoglyerol, PG 16:1/18:3. Similarly, 2 other molecular species of PG in table 2 have been determined.

Discussion

By using HPLC-HRMS technique to analyze green algae *H. incrassata*, 2 groups including PI (3 molecular species) and PG (4 molecular species) in the phospholipid subclass have been identified, without the appearance of phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS), which is similar to other reports of green algae [12]. It may be related to the characteristic of the algae about $CaCO_3$ synthesis. Previous studies [12–15] have demonstrated that the diacylglyceryltrimethylhomoserine (DGTS) group belonging to the betaine lipid subclass can substitute for PC at the chloroplast membrane. Thus, the absence of PC in this seaweed agrees with our recent publication about the betaine lipid subclass of the *H. incrassata* species which has detected only one DGTS group with 17 molecular species.

In 7 molecular species identified, fatty acids containing 16 carbons (C16) are the main composition of both PG and PI groups (9/14 acyl radicals). By the GC analysis of the composition and content of fatty acids, C16 fatty acids were detected with various contents in green algae *H. incrassata*, which is similar to the report of Victor et al., on the fatty acid composition of 26 seaweed species including 8 green algae [13]. Regarding the composition of fatty acids in different phospholipid subclasses, the PI group (table 2) is formed mainly from two saturated fatty acids C16:0, C18:0 and unsaturated fatty acids C20:4. These C20:4 fatty acids are important in the composition of some prostaglandins in order to control the inflammation [16, 17]. In contrast, the PG group (table 3) is formed by only one saturated fatty acid C16:0 and unsaturated fatty acids C16:1, C18:2, C18:3. Notably, C18:2 and

C18:3 acids appear on the fatty acid composition of this species by the GC analysis in the forms of C18:2n-6 and C18:3n-3, which are the important fatty acids for the synthesis process in omega-3 and omega-6 disorder to endothelial cell and nervous system balance [18, 19]. It is remarkably related to bioactivities of these compounds and the function of phospholipid subclass, suggesting further studies about effects on the structure and activities of phospholipid species.

CONCLUSION

By using HPLC-HRMS to analyze green algae *H. incrassate*, this study has identified 7 molecular species of phospholipid belonging to the PI group (3 species) and the PG group (4 species). Amongst those, PI 32:0 (16:0/16:0) has the highest content of 52.03% and PG 34:3 (16:1/18:2) has the highest content of 61.02%.

The compositions of fatty acids in the phospholipid subclass are mainly C16, which is rich in the green algae total lipid, but there is a difference between two groups of phospholipid molecular species. While the PI group mainly contains two saturated fatty acids (C16:0, C18:0) and 1 unsaturated fatty acid C20:4, PG has only 1 saturated fatty acid C16:0 and three unsaturated fatty acids (C16:1, C18:2, C18:3).

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REFERENCES

[1] Dam, D. T., 2008. Initial research on the distribution and coverage of some calcified seaweed species in Truong Sa archipelago, Vietnam Sea. *Vietnam Association of Marine Science and Technology*, *10*, 18–22.

[2] Tien, D. D., 2016. Species composition and distribution of seaweeds from some small islands (Nam Yet, Son Ca, Song Tu Tay, Sinh Ton) of Truong Sa archipelago. *Vietnam Journal of Marine Science and Technology*, *16*(3), 297–305. https://doi.org/10.15625/1859-3097/16/3/8124.

[3] Carneiro, P. B. D. M., Pereira, J. U., and Matthews-Cascon, H., 2018. Standing stock variations, growth and $CaCO_3$ production by the calcareous green alga Halimeda opuntia. *Journal of the Marine Biological Association of the United Kingdom*, *98*(2), 401–409. https://doi.org/10.1017/S0025315416001247.

[4] Silva, A. M. O., Novoa, A. V., Gutierrez, D. D., and Mancini-Filho, J., 2017. Seaweeds from Halimeda genus as sources of natural antioxidants. *J. Anal. Pharm. Res.*, *5*(6), 1–5.

[5] Novoa, A. V., Andrade-Wartha, E. R., Linares, A. F., Genovese, M. I., González, A. E. B., Vuorela, P., Costa, A., and Mancini-Filho, J., 2011. Antioxidant activity and possible bioactive components in hydrophilic and lipophilic fractions from the seaweed Halimeda incrassata. *Revista Brasileira de Farmacognosia*, *21*(1), 53–57. https://doi.org/10.1590/S0102-695X2011005000010.

[6] El Gamal, A. A., 2010. Biological importance of marine algae. *Saudi Pharmaceutical Journal*, *18*(1), 1–25. https://doi.org/10.1016/j.jsps.2009.12.001.

[7] La Guardia, M., Giammanco, S., Di Majo, D., Tabacchi, G., Tripoli, E., and Giammanco, M., 2005. Omega 3 fatty acids: biological activity and effects on human health. *Panminerva Medica*, *47*(4), 245–257.

[8] Calder, P. C., and Yaqoob, P., 2009. Omega-3 polyunsaturated fatty acids and human health outcomes. *Biofactors*, *35*(3), 266–272. https://doi.org/10.1002/biof.42.

[9] State Committee for Science and Technology, 1981. Temporary rules of marine integrated investigation (section Seaweed). *Science and Technics Publishing House, Hanoi*. 205 p.

[10] Bligh, E. G., and Dyer, W. J., 1959. A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology*, *37*(8), 911–917. https://doi.org/10.1139/059-099.

[11] Pham, T. H., Nguyen, V. T. A., Do, T. T. T., Do, A. D., Dam, D. T., Tran, T. T. V., Pham, Q. L., and Le, T. T., 2020. Lipidomics and Anti-Inflammation Activity of Brown Algae, *Lobophora* sp., in Vietnam. *Journal of Chemistry, 2020*. https://doi.org/10.1155/2020/8829054.

[12] Vaskovsky, V. E., Khotimchenko, S. V., Xia, B., and Hefang, L., 1996. Polar lipids and fatty acids of some marine macrophytes from the Yellow Sea. *Phytochemistry*, *42*(5), 1347–1356. https://doi.org/10.1016/0031-9422(96)00117-3.

[13] Thompson Jr, G. A., 1996. Lipids and membrane function in green algae. *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism*, *1302*(1), 17–45. https://doi.org/10.1016/0005-2760(96)00045-8.

[14] Harwood, J. L., 1998. Membrane lipids in algae. In *Lipids in photosynthesis: structure, function and genetics*, pp. 53–64. *Springer, Dordrecht*.

[15] Dembitsky, V. M., and Rezanka, T., 1995. Distribution of acetylenic acids and polar lipids in some aquatic bryophytes. *Phytochemistry*, *40*(1), 93–97.

[16] Thanh Le Tat, 2015. Filter research, isolation, and identification of active fatty acid, arachidonic acid and prostaglandin from red seaweed. *PhD thesis, GUST*. 140 p.

[17] Sakai, M., Kakutani, S., Tokuda, H., Suzuki, T., Kominami, M., Egawa, K., Saito, K., Rogi, T., Kawashima, H., Shibata, H., and Sasaki, S., 2014. Arachidonic acid and cerebral ischemia risk: a systematic review of observational studies. *Cerebrovascular Diseases Extra*, *4*(3), 198–211. https://doi.org/10.1159/000367588.

[18] Pham Q. L., Chau V. M., 2005. Biologically active lipids and fatty acids from nature. 1st Ed. *Science and Technics Publishing House, Hanoi*. pp. 24–30.

[19] Burri, L., Hoem, N., Banni, S., and Berge, K., 2012. Marine omega-3 phospholipids: metabolism and biological activities. *International Journal of Molecular Sciences*, *13*(11), 15401–15419. https://doi.org/10.3390/ijms131115401.