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Application News

Phospholipid Profiling of *Litopenaeus Vannamei* Raised in Different Environments

Masaki Yamada, Naoko Nagano

User Benefits

- The MRM Library for Phospholipid Profiling enables quantitative phospholipid analysis of any sample.
- A phospholipid profiling method for *vannamei* shrimp was developed to detect 135 phospholipids.
- Quantitative comparisons focusing on the fatty acids bound to phospholipids allow the identification of phospholipids specific to raising environments.
- The Multi-omics Analysis Package supports searching for components with significant differences in contents.

■ Introduction

Litopenaeus vannamei is a shrimp species rich in nutrients, including omega-3 fatty acids and functional phospholipids such as phosphatidylserine. More than 8 million tons of *vannamei* shrimp are produced worldwide, about half of which comes from aquaculture. In this article, the differences in phospholipid profiling of *vannamei* shrimp depending on the raising environment are introduced.

The MRM library for Phospholipid Profiling (Shimadzu Corporation) is capable of profiling 867 components of phospholipids by taking the combinations of 17 fatty acids into account. Phospholipid profiling of *vannamei* shrimps raised in three different environments was performed, and 135 phospholipid species in total were detected. Multivariate analysis and statistic analysis revealed several phospholipids that differed significantly by raising environment.

■ Sample and Pretreatment

Vannamei shrimp with three raising environments, A, B, and C shown in Fig. 1, were provided by the Central Research Laboratory, Nissui Corporation. Samples of about 100 mg of the white meat portion were cut from the frozen shrimps (Table 1). A mixed solution of methanol and butanol was added at the rate of 100 mg/mL, and the lipid was extracted by crushing using a bead crusher. After being centrifuged at 13,000 rpm for 15 min at 4 °C, the supernatant was diluted by a factor of 10 by volume with methanol, and subjected to LC/MS analysis.

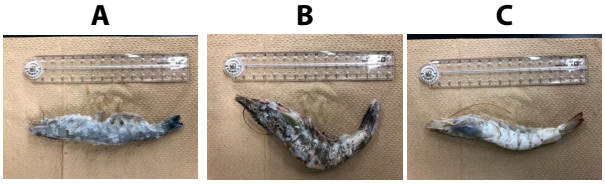

Fig. 1 *Vannamei* Shrimp from Three Raising Environments

Table 1 *Vannamei* Shrimp Samples

Raising Environment	No.	Mass (Whole Shrimp) (g)	Mean (g)	Sample Mass (mg)	Solvent Added (µL)
A	A-1	28.7	26.9	113	1130
	A-2	26.9		105	1050
	A-3	25.6		108	1080
	A-4	28.0		113	1130
	A-5	25.5		117	1170
B	B-1	49.6	43.1	117	1170
	B-2	43.2		104	1040
	B-3	36.3		110	1100
	B-4	45.7		117	1170
	B-5	40.8		110	1100
C	C-1	26.5	25.1	120	1200
	C-2	25.3		112	1120
	C-3	25.2		110	1100
	C-4	25.7		111	1110
	C-5	22.9		109	1090

Hereafter, sample A, sample B, and sample C refer to any of the samples from raising environment A, raising environment B, and raising environment C, respectively.

Table 2 Analytical Conditions of LC/MS

[HPLC Conditions] (Nexera™)	
Column:	Phenomenex Kinetex C8 (150 mm × 2.1 mm, 2.6 µm)
Column Oven:	50 °C
Solvent A:	20mM Ammonium formate - water
Solvent B:	Acetonitrile/2-Propanol (1/1, v/v)
Flowrate:	0.4 mL/min
Injection Volume:	1 µL
[MS Conditions] (LCMS-8050)	
Ionization:	ESI, Positive/Negative
Mode:	MRM
Nebulizing Gas Flow:	2.0 L/min
Drying Gas Flow :	10.0 L/min
Heating Gas Flow :	10.0 L/min
DL Temp.:	250 °C
Block Heater Temp.:	400 °C
Interface Temp.:	150 °C
CID Gas Pressure:	270 kPa
Dwell Time/Pause Time:	1 msec/1 msec

■ Analytical Conditions

The analytical conditions for HPLC and MS are shown in Table 2. The flowrate and oven temperature were modified from the default conditions of the MRM Library for Phospholipid Profiling so that an analysis is completed in 20 minutes. The interface temperature of the MS conditions was set to 150 °C, which is lower than the default value.

■ Developing MRM Transitions for Phospholipid Profiling

The MRM Library for Phospholipid Profiling contains 1969 MRM (Multiple Reaction Monitoring) transitions for monitoring 867 phospholipids. Of these, 422 are MRM transitions that monitor product ions generated by the elimination of polar head groups (PH-MRMs), and the others are MRM transitions that monitor deprotonated fatty acids (FA-MRMs) in negative ion mode.[1] The MRM Library for Phospholipid Profiling can be adopted for any samples like blood, tissue, and cultured cells, and can be analyzed for isomers of diacylphospholipid fatty acids. Instead of analyzing the sample with all 1969 MRM transitions, the sample was first analyzed with 422 PH-MRMs, and the FA-MRMs for fatty acid determination were combined for the detected peaks to create a 2nd method.[2] In this study, the 2nd method was created consisting of 833 MRM transitions from the analysis of a lipid extract of the *vannamei* shrimp. The results of the analysis of the 15 samples in Table 1 using the 2nd method are described below.

■ Number of Detected Phospholipids

A total of 135 phospholipids was detected when samples from the three raising environments were analyzed by the 2nd method. These included 52 phosphatidylcholine (PC), 50 phosphatidyl-ethanolamine (PE), 9 phosphatidyl-serine (PS), 8 lyso-phosphatidylethanolamine (LPE), 6 sphingomyelin (SM), 5 lysophosphatidylcholine (LPC), and 5 lysophosphatidyl-inositol (PI).

■ Peak Identification

For phospholipids other than SM, peaks were identified because of the fact that they were detected at the same retention time in both PH-MRM and FA-MRM chromatograms. LPC and LPE were judged as "Not Detected" if no peak was detected by FA-MRM. An example of peak identification for diacyl phospholipids is shown in Fig. 3. Peak (1) is identified as PC 16:0_20:4, with a peak detected at 8.38 minutes in the black line (782.55 > 184.1) indicating the polar group choline, the pink line indicating the fatty acid 16:0, and the blue line indicating the fatty acid 20:4. Similarly, peak (2) detected at 8.10 minutes can be identified as PC 18:2_18:2. Peak (3) was identified as PC 18:1_18:3 because a peak was detected at 8.13 minutes in the line of fatty acid 18:3 and the peak at 8.10 minutes in the black line was slightly tailed. No peaks were detected in the MRM chromatograms (brown and green) for PC 16:1_20:3. The peak detected at 7.98 minutes in the black line (*) is considered to be the +2 isotope peak of PC 36:5. Although the data are not shown, PC 16:0_20:5 was detected at 7.98 minutes, and the peak had the sixth largest area of the 52 PC species (Fig. 4).

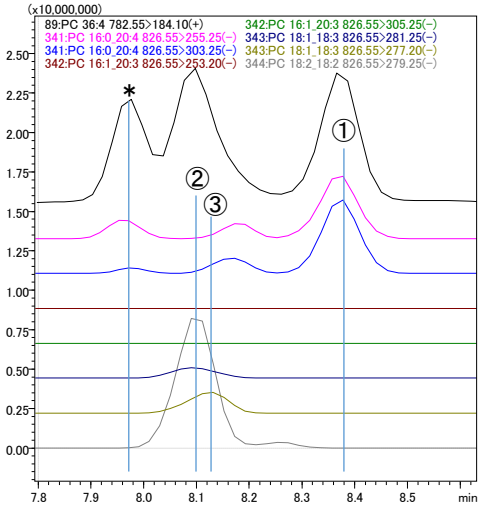

Fig. 3 MRM Chromatograms of PC 36:4
Except for the black line 782.55 > 184.1, the intensity of the vertical axis was magnified by a factor of 500. The sample is a shrimp raised in environment A.

■ Phospholipid Profiling

Table 3 shows the sum of the peak areas detected by MRM in negative ion mode for all 135 components detected from three raising environments. Although the amount of extraction solvent added per unit tissue mass was made uniform (Table 1), the sum of peak areas differed by up to 8 % between the raising environments.

Table 3 Mean of Total Peak Areas Detected by MRM in Negative Ion Mode, Monitoring Product Ions Derived from Fatty Acids for the 135 Components, Relative Area%, and Standard Error (n = 5)

	A	B	C
Mean of Total Area	10563996	10253754	9755261
Relative Area (%)	103	100	95
Standard Error	136830	236380	188748

The phospholipid profiling of the 52 PC species is shown in Fig. 4. The average peak area of each lipid (n = 5) is shown on the horizontal axis and expressed as a logarithm. The 40 phospholipids containing DHA 22:6 or EPA 20:5 are highlighted in blue. Eleven PC species contained DHA or EPA, and the areas of sample A were relatively large, especially in PC 16:0_22:6 where the area of sample A was 2 to 3 times that of samples B and C. On the other hand, the area of sample A was less than half that of sample B and sample C in the five PC species shown in gray, including the fatty acid 20:2.

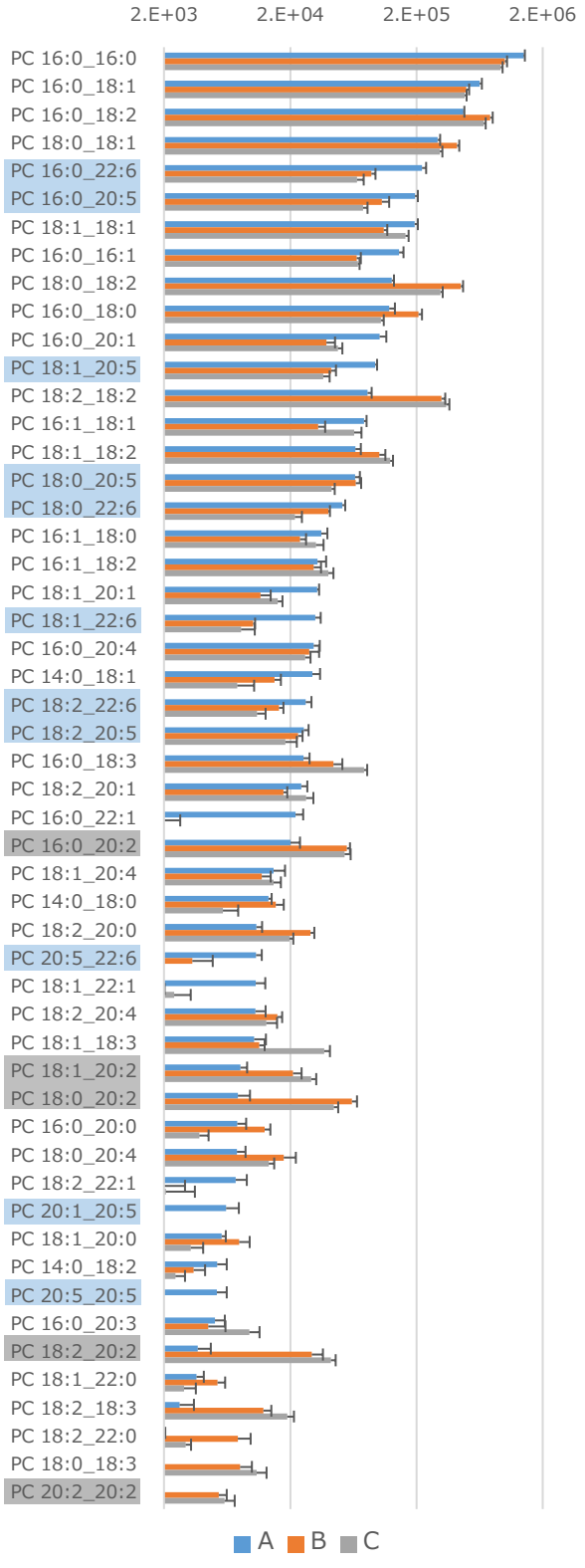

Fig. 4 Peak Area of the 52 PCs
From the top, PC species are listed in the order of peak area of sample A, and the area 2,000 to 2,000,000 is expressed as a logarithm. Error bars are standard errors (n = 5).

The phospholipid profiling of the 50 PE species is shown in Fig. 5. Of the 50 species, 22 contain DHA or EPA, which is relatively high in sample A. In particular, PE 20:5_20:5 had more than 2 times the area in sample A than the other 2 samples, and PE 16:0_22:6, PE 18:1_22:6, and PE 16:0_20:5 had 1.5 to 2 times the area in sample A than the other 2 samples. Fig. 6 shows the profiling of the other 33 components. Regarding PS and PI, it was found that 6 out of 14 species contain DHA or EPA, and they had a relatively small area in sample C. Lyso-phospholipids tended to have a smaller peak area in sample B than in other samples.

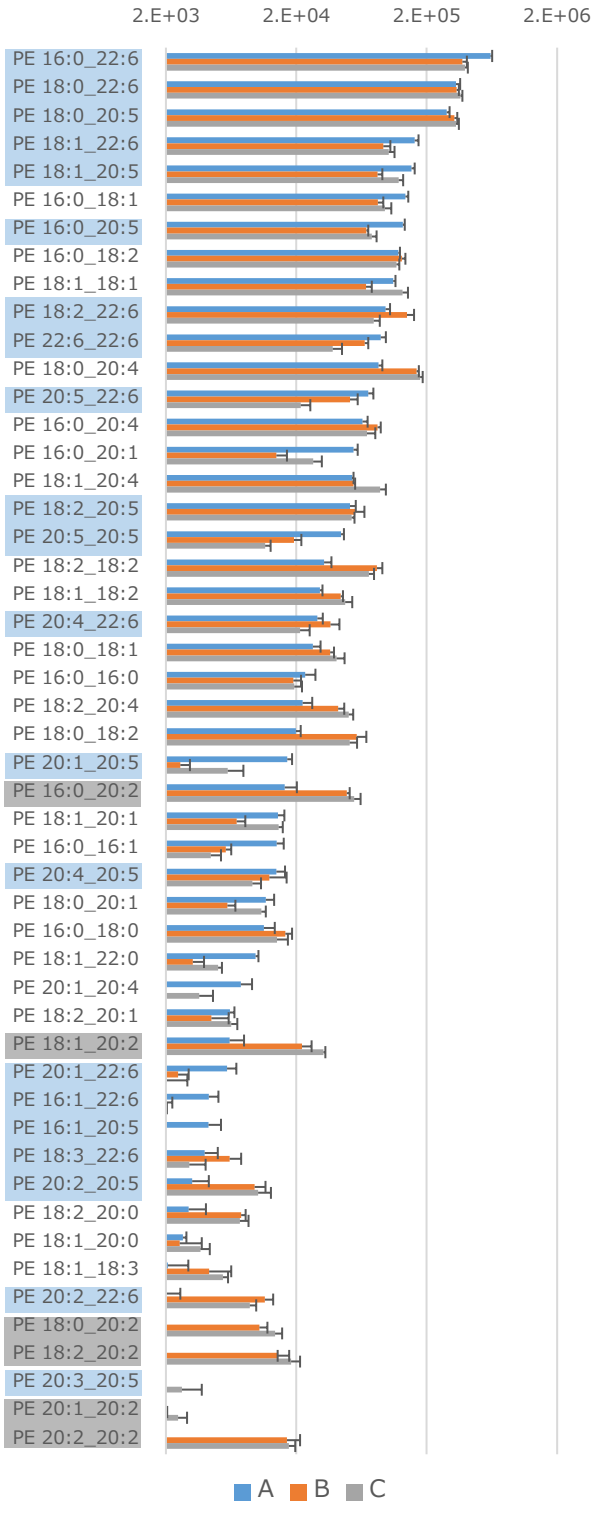

Fig. 5 Peak Area of the 50 PE Species
Area 2,000 to 2,000,000 is expressed as a logarithm. Error bars are standard errors (n = 5).

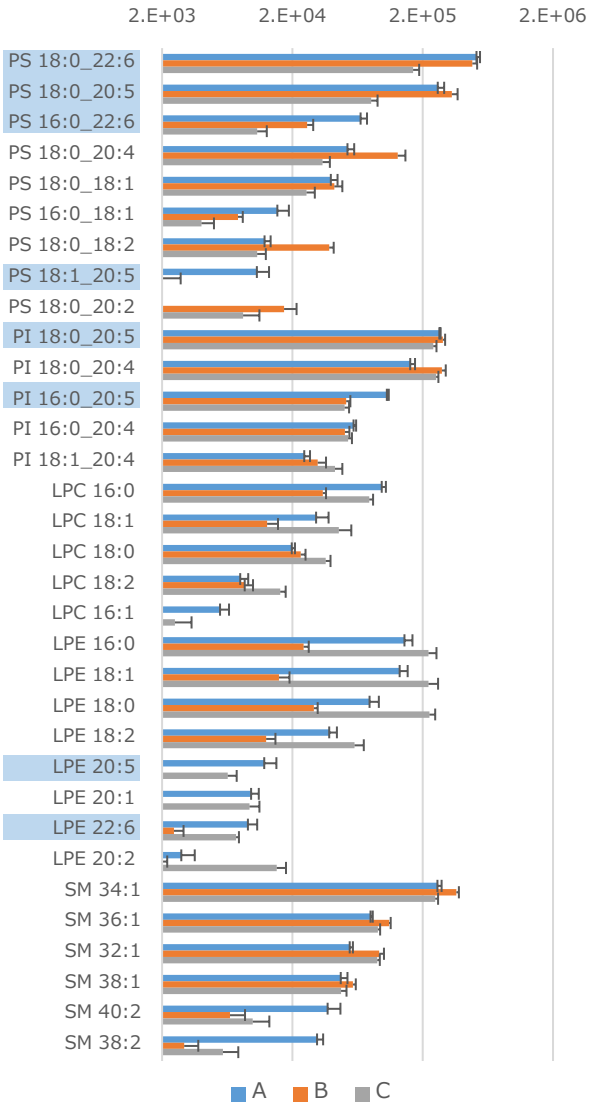

Fig. 6 Peak Area of PS, PI, LPC, LPE, and SM Species
Area 2,000 to 2,000,000 is expressed as a logarithm. Error bars are standard errors (n = 5).

■ Statistical Analysis

Fig. 7 shows the results of multivariate analysis using the Multi-omics Analysis Package. The score plots were clearly differentiated by according to the raising environment.

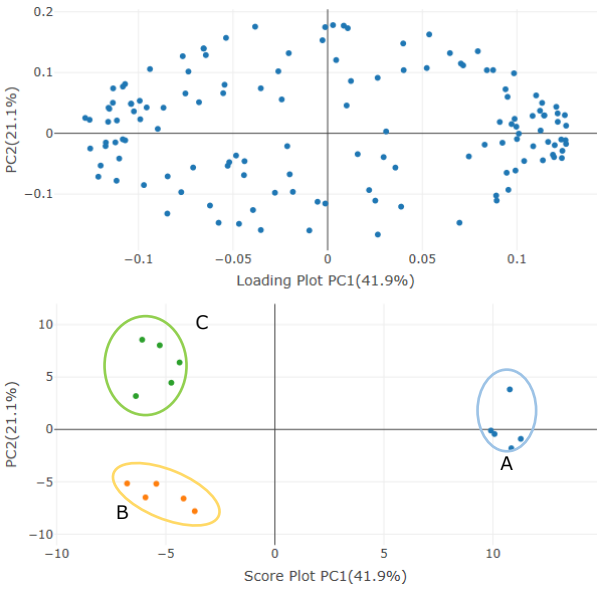

Fig. 7 Loading Plot and Score Plot

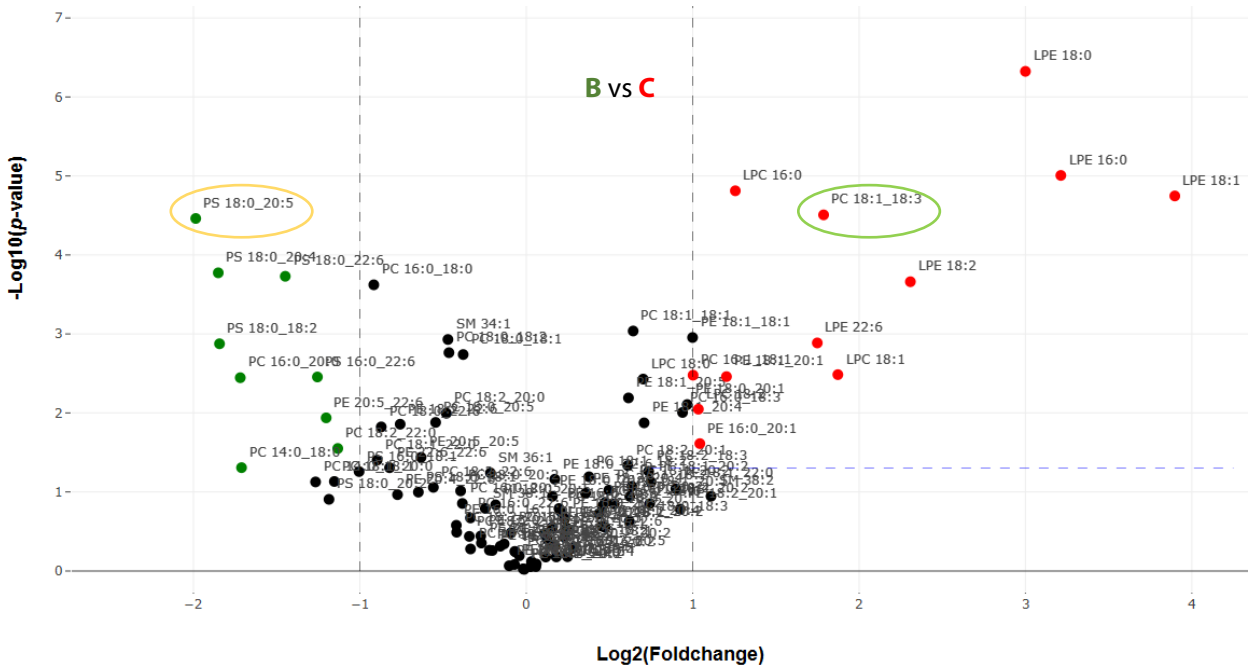

Fig. 8 Volcano Plot of Sample B and C
Green are the plots with large areas in sample B, and red are those with large areas in sample C.

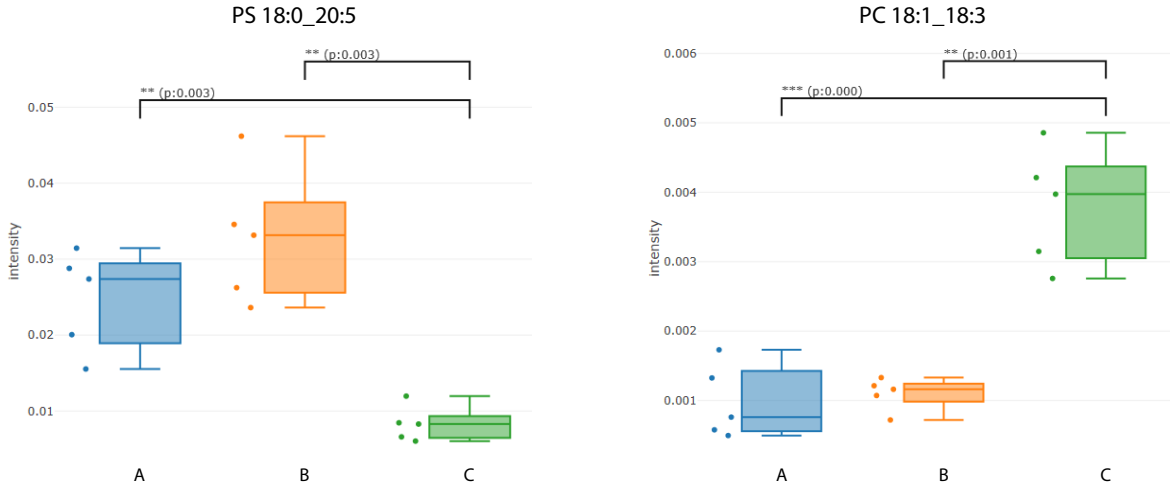

Fig. 9 Box plot of PS 18:0_20:5 and PC 18:1_18:3

Volcano plot analysis was performed to find significant differences between samples B and C (Fig. 8). The components shown in green are two or more times more abundant in sample B, with the *p*-value of significance less than or equal to 0.05. The components shown in red are two or more times more abundant in sample C. PS was found to be a significantly abundant component in sample B. LPE and LPC were found to be abundant in sample C. Fig 9 shows a box plot of PS 18:0_20:5, which is abundant in sample B, and PC 18:1_18:3, which is abundant in sample C.

■ Conclusion

- ✓ A 20-minute cycle phospholipid profiling method has been developed for *vannamei* shrimp.
- ✓ In total, 135 phospholipids including isomers with different fatty acid combinations were detected.
- ✓ Focusing the fatty acids bound to phospholipids enabled several phospholipids specific to the raising environment to be found.
- ✓ Statistical analysis with the Multi-omics Analysis Package helped to find significant differences in the phospholipids.

■ Reference

1) Masaki Yamada, Shimadzu Review, 77 (3-4), 125-134 (2020).
2) Japanese Patent No. 06611009.

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