Multivariate analysis for molecular species of cholesteryl ester in the human serum

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Abstract

Cholesteryl ester (CE) is an ester of cholesterol and fatty acid (FA). Plasma CE reflects complicated metabolisms of cholesterol, phospholipids, lipoproteins, and dietary FAs. Informatics approach could be useful for analysis of CE species. In this study, two basic dimension reduction methods, principal component analysis (PCA) and factor analysis, were applied to serum CE species determined by LC/MS/MS in a Japanese population (n=545). PCA and factor analysis both reflected size (concentration), food source, fat solubility, and biological aspect of CE species. In comparison between PCA (PC4) and factor analysis (factor 4), the latter was found more suggestive for a biological aspect of n-6 FAs. Cholesteryl docosahexaenoate (DHA) was found unique by factor analysis, possibly relevant to the unique accumulation of DHA in the brain. Informatics approach, especially factor analysis, might be useful for analysis of complicated metabolism of CE species in the serum.

Keywords: factor analysis, principal component analysis, epidemiology, LC/MS/MS, cholesterol, fatty acid.
Introduction

Multivariate statistical approach has become important in analytical and clinical chemistry, especially for analyses of large and complicated biological or medical datasets generated from comprehensive mass spectrometry.\textsuperscript{1} This is also true in recent lipid research. For instance, lipidomics coupled with multivariate analysis has been reported as a powerful tool for discovery of potential biomarkers of cancers.\textsuperscript{2,3}

**Cholesteryl ester (CE)** is the ester of cholesterol and fatty acid (FA). Because of the molecular diversity of FAs, there are various kinds of CE species in human serum, where CE serves as the major core lipid of low-density lipoproteins (LDL) and high-density lipoproteins (HDL).\textsuperscript{4} In clinical laboratories, the concentration of each CE species is not available since CE is obtained as total esterified cholesterol by subtracting free cholesterol from total cholesterol both enzymatically determined.\textsuperscript{4} For this reason, only a limited number of literatures have reported the concentrations of CE species in a large population, and moreover, few of them contained multivariate analysis.

Warensjö et al. studied the average proportions, not absolute quantities, of FA species in serum CE using gas chromatography coupled with thin-layered chromatography in the men at ages of 50 and 70 years with and without the metabolic syndrome.\textsuperscript{5} The authors conducted principal component analysis (PCA), and found significant relationship between the onset of metabolic syndrome and the factors of linoleic acid (FA 18:2) and n-3 polyunsaturated FAs. Including their studies, the concentration of CE species has been commonly determined by time-consuming and laborious gas chromatography for methylated or silylated FAs, following to chromatographic separation of CE fraction from other lipid classes.\textsuperscript{6,7}

In our present study, we determined absolute concentrations of each CE species in the serum for a general Japanese population using liquid chromatography/tandem mass spectrometry (LC/MS/MS) with the use of $^{2}$H$_{3}$-labeled internal standards (IS) for each CE species.\textsuperscript{8,9} To analyze
the complex CE dataset, we employed an informatics approach, that is, multivariate analysis including dimension reduction methods. The aim of this report is presenting and discussing the results of our multivariate analysis for the serum CE dataset. Informatics approach might be useful to reach better understanding of the metabolisms and biological roles for each CE species.

Experimental

Chemicals and reagents

Cholesterol and all LC/MS grade solvents including methanol, 2-propanol, n-hexane, and water purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Ammonium acetate was purchased from Sigma-Aldrich (St. Louis, MO, USA). Other chemicals and reagents were purchased from Kanto Chemical Industry (Tokyo, Japan). Palmitic acid (FA16:0), stearic acid (FA18:0), oleic acid (FA18:1), FA18:2, linolenic acid (FA18:3), and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Arachidonic acid (FA20:4) and docosahexaenoic acid (FA22:6) were purchased from Sigma-Aldrich Co., LLC. (MO, USA). Both CE and \( ^2\text{H}_3\)-CE (IS) were prepared as described previously, and were stored stable at \(-80\, ^\circ\text{C}\) for at least 3 years.

Blood samples

Blood samples were obtained after an overnight fast from the persons (n = 545; 300 women and 245 men; aged 35 to 79 years old) who participated in general health examinations in the year of 2015 in Suttsu town of Hokkaido, Japan. Suttsu town has a population of 3,100 approximately, and its main industry is fishery. After blood coagulation at room temperature, serum was separated by centrifugation at 4\(^\circ\text{C}\), and stored at -80\(^\circ\text{C}\) for no longer than 3 years before analysis. The samples were stable at this conditions. Ethical approval was obtained by the ethic committee of the Faculty of Medicine (15-002, 16-007) and the Faculty of Health Sciences (16-10), Hokkaido
University, and informed consent was obtained from each participant.

**LC/MS/MS**

The detailed conditions LC/MS/MS for CE species and the results of validation studies were reported previously. Briefly, the assay was conducted using a TSQ Quantum Access MAX (Thermo Fisher Scientific, Inc., Walthum, MA, USA) linked to a Thermo Finnigan Surveyor HPLC System. LC separation was carried out on an Accucore C18 (Thermo Fisher Scientific, Inc.) at 40°C. MS detection was implemented in a selected reaction monitoring (SRM) mode under condition of atmospheric pressure chemical ionization in positive ion mode. To determine the calibration curves, the mix standards of CE solution with 0.01, 0.04, 0.2, 1, 2, 4, 10, and 20 μmol/L were prepared. Each standard solution contained 1 μmol of the 3H3-CEs. The integration of the peak area and the plotting of each calibration curve were carried out using Xcalibur 2.0.7.

Serum sample (20 μL), added with 12 μmol/L 3H3-labeled CEs 16:0, 18:0, 18:1, 18:2, 18:3, 20:4, 20:5, and 22:6 as internal standards, was mixed with ethanol (200 μL), hexane (1200 μL), and deionized water (1,000 μL). Then, centrifugation (1,500×g, 10 min) was conducted to separate the organic layer and the aqueous layer. The collected organic layer was concentrated in a centrifugal evaporator, and redissolved in isopropanol (300 μL). The sample was then diluted 8-fold with isopropanol, and 5 μL was used for LC/MS/MS. Fine separation of all CE species and excellent results of validation studies were reported previously. CVs and recoveries ranged 1.6% - 6.6%, and 94.4% - 107.2%, respectively.

**Sample pretreatment**

Serum (20 μL) was mixed with ethanol (200 μL) containing a mixture of ISs (1.2 nmol each) and added with hexane (1200 μL) and distilled water (1000 μL). After centrifugation at 1500×g for 10 min, the organic layer was collected and dried under vacuum (Tomy centrifugal...
concentrator, Tokyo, Japan). Then, the residue was dissolved in isopropanol (300 μL) and filtered using a centrifugal filtering device (PVDF 0.1 μm; Merck Millipore Ltd., Carrigtwohill, Ireland). Finally, the sample was then diluted 8-fold with isopropanol, and 5 μL was injected to LC-MS/MS.

**Data processing**

The integration of the peak area and the plotting of each calibration curve using 1/x weighting were performed by Xcalibur 2.0.7. (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

**Dataset**

The CE dataset contained 8 subtypes of CE molecules: cholesteryl palmitate (CE 16:0), cholesteryl stearate (CE 18:0), cholesteryl oleate (CE 18:1), cholesteryl linoleate (CE 18:2), cholesteryl linolenate (CE 18:3), cholesteryl arachidonate (CE 20:4), cholesteryl eicosapentaenoate (CE 20:5) and cholesteryl docosahexaenoate (CE 22:6).

**Statistical analyses**

Dimension reduction methods, namely, PCA and factor analysis were carried out. The statistical analysis was performed with the use of “psych” package in R (version 3.5.2).

**PCA**

PCA is the most basic dimension reduction method. In this method, data is transformed into new space where variables are uncorrelated with others. This new space is transformed from the observed data by orthogonal transformation based on eigenvalue of the observed data’s covariance or correlation matrix. The main purpose of PCA is to find major information from multivariate observed data and represent the interrelationship between variables, which means there is no need to use all the principal components of the sample data but the first \( m \) principal
components, whose cumulative variance or correlation coefficients usually occupying 80 percent with the corresponding first $m$ eigenvalues.\(^{11}\)

**Factor analysis**

Factor analysis is a statistic method to explain variability among a large set of observed, correlated variables. In this method, the described variabilities are from a smaller set of latent factors to reflect variation of observed variables. In factor analysis, we used maximum likelihood factor analysis to explain the characteristics of CEs.

In factor analysis, uniqueness is calculated as a proportion of variance of the variable not associated with the common factors. Uniqueness of one variable displays how its variance to the variable not shared with other variables.\(^{12}\)

To judge the goodness of fit, we used a combination of two criterions: The Root Mean Square Error of Approximation (RMSEA) and Bayesian information criterion (BIC) to decide the factor number in modeling. The RMSEA was a chi-square-based calculation for each simulation. It was thought that RMSEA value of between 0.08 and 0.1 indicated a mediocre fit, and below 0.08 indicated a good fit. BIC value is a maximum-likelihood-based method for model selection. In this method, model with the lowest BIC value is preferred.\(^{13}\)

**Results and Discussion**

**PCA of CEs**

PCA was applied on the CE dataset to obtain a general view on CEs. There was no gender effect in the concentrations of CE species (Table 1). However, possible age effect was suggested typically in CE 20:5 (Table 1). Therefore, we made scatter plot with biplot using color codes for age groups (Figs. 1 - 4).

The biplot of the first (PC1) and the second (PC2) principal components displays that larger
loading values in PC1 were obtained for the group of CEs 16:0, 18:1, 20:4, 18:2, and 18:3. The smaller loading values were obtained for another group of CEs 22:6, 20:5, and 18:0 (Fig. 1). The separation of two groups is consistent with the difference in the CE concentrations for the total, except for CE 18:3 (Table 1). Thus, we considered PC1 as the size factor. In PC2, CEs were separated into three groups (Fig. 1). The first group showed positively larger loading values and contained CEs 18:2, 18:3, and 18:1. The second group showed absolutely smaller loading values and contained CEs 18:0, 16:0 and 20:4. The third group showed negatively larger loading values and contained CEs 22:6 and 20:5. Thus, we thought it was also likely to consider PC2 as the reflection of source factor: the first, second, and third groups corresponded to the plants, meats, and fish, respectively (Fig. 1).14

The biplot of the third (PC3) and the forth (PC4) principal components displays that positively large loading values were obtained for the group of CEs 18:0 and 16:0 in PC3 (Fig.2). The negatively small loading values were obtained for CEs 22:6, 18:1, 18:2, 18:3 and 20:4. Whereas, the negatively large loading values are obtained for CE 20:5. CE 18:0 and 16:0 are saturated CEs, the rest CEs are unsaturated CEs. Saturated CEs have higher fat solubility than unsaturated CEs.15 Therefore, PC3 was considered as representation of saturation or fat solubility. In PC4, negatively large loading value was obtained for CE 18:3, and positively large loading value was obtained for CE 20:4 and 18:2. CE 18:3 belongs to \( \mathbf{n} \)-3 group, which decreases inflammation.16 Meanwhile, CE 20:4 and 18:2 belong to \( \mathbf{n} \)-6 group, which increases inflammation. It is, however, unlikely that PC4 represents inflammation effect. CEs 22:6 and 20:5 showed only small loading values in PC4 in spite that they are among \( \mathbf{n} \)-3 group and reduce inflammation more effectively than CE18:3. Thus, PC4 remains to be an obscure axis in this study, suggesting PC4 as a reflection of multiple factors. In conclusion, PCA was found useful, but its utility was not enough for giving a precise explanation for PC4 due to complication of multiple principal components. We considered that the main solution to avoid an obscure axis was to give a specific factor number in modeling.
Therefore, we applied factor analysis with a selected factor number for further understanding on the CE dataset.

**Factor analysis of CEs**

**Decision of factor number**

In factor analysis, factor number is determined by a combination of two criterions: RMSEA index, and lowest BIC value. In application on the CE dataset: the factor number decided by RMSEA index, and lowest BIC value were 4 (Table 2), and we certainly can explain the model better than models with other number. Therefore, we chose the model with 4 factors.

**Results of factor analysis**

In the result of factor analysis, all loading values were positive, and therefore, the factor pattern was simple and easy to interpret. The subtypes with the loading values no less than 0.60 were generally considered to be significant. The biplot of factors 1 and 2 displays that the CE subtypes with the loading values ≥ 0.6 were CEs 18:1, 18:3, 18:2, 20:4 and 16:0 in factor 1 (Fig.3). Factor 1 was thought to be the size factor with the same reason mentioned in PC1 analysis (Table 1). In factor 2, CEs 22:6 and 20:5 showed the large loading values ≥0.6. We considered factor 2 as a reflection of the fish intake because CEs 22:6 and 20:5 are usually obtained from fish oil. On the other hand, we also found that subtypes were also divided into three groups. CEs 20:4, 16:0, 18:0, and 18:1 were in group 1, which are mainly obtained from animal oil. CEs 18:2 and 18:3 were in group 2, which are mainly obtained from plants oil. CEs 22:6 and 20:5 were in group 3, which are mainly obtained from fish oil. Therefore, factor 2 was also likely to reflect food source of FAs.14

In the biplot of factor 3 and 4, the subtypes with the large loading values ≥ 0.5 in factor 3 were CEs 16:0 and 18:0, both of which were with saturated FAs (Fig.4). On the other hand, the rest of
CEs showing the small loading values were those with unsaturated FAs. Therefore, we considered factor 3 to reflect fat solubility with the same reason in PC3 analysis. In factor 4, only CE 20:4 showed a large loading value (Fig.4). FA 20:4 (arachidonic acid) is metabolized to eicosanoids (prostacyclin, thromboxanes, leukotrienes, and prostaglandins) that are signaling molecules and play an important role in mediating inflammatory responses, exerting a wide spectrum of biologic actions in various body systems.\textsuperscript{15} In contrast, CEs 20:5 and 22:6 showed the smaller loading values than CE 20:4. FAs 20:5 (EPA) and 22:6 (DHA) are metabolized to proresolving mediators, respectively, resolving and protectin.\textsuperscript{16} Therefore, we consider that factor 4 might reflect inflammatory aspect of CEs.

**Summary and general consideration**

First, the order of the CE species in PC1 and factor 1 were parallel to the order of the concentrations of CE species, suggesting PC1 and factor 1 serve as the size factors (Table 1). The PC1, PC2, and PC3 in PCA, and also the factor 1, factor 2, and factor 3 in factor analysis well reflected the size, the food source (or fish intake), and the fat solubility, respectively (Figs. 1 and 2). In comparison between PC4 and factor 4, however, only factor 4 reflected the inflammation and signaling, whereas PC4 failed to do that. It means that factor analysis performed better than PCA in this study.

In factor analysis of the CE dataset, the uniqueness value of CE 22:6 was remarkably high among those of CEs. In contrast, the uniqueness of free FA 22:6 and total FA 22:6 were not so high (Table 3). Furthermore, another n-3 FA-containing CE, namely, CE 20:5 was the lowest in the uniqueness value among all CEs (Table 3). Uniqueness contains two parts: specific variance and error variance. Specific variance is specific to a particular item. Error variance is from errors of measurement.\textsuperscript{12} In the present case, the accuracy and precision of the measurement of CE 22:6 was confirmed in the validation studies. Therefore, the observed high uniqueness of CE 22:6
should largely reflect the specific variance. These findings might indicate the possibility of unique metabolic pathway for CE 22:6. FA 22:6 is enriched in the brain, representing > 40% of total brain PUFA.\textsuperscript{17} Since the synthesis of FA 22:6 from the n-3 precursor, α-linolenic acid, is low in the brain, a continuous supply of FA 22:6 across the blood-brain barrier is required. In plasma, FA 22:6 can be in several fractions, such as phospholipids, lysophospholipids, CEs, TGs, and free FAs.\textsuperscript{17} It is of our interest that lyosphosphatidylcholine 22:6 is transported from plasma to the brain and the eye by a specific transporter, namely Mfsd2a.\textsuperscript{19,21} Also, it is reported that FA binding protein 5 (FABP5) mediates the transport of free FA 22:6 across the blood-brain barrier.\textsuperscript{18} The high uniqueness value for CE22:6 might suggest a possibility of a unique transport system for CE 22:6 from plasma to the brain, in spite that CE 22:6 is a minor part (< 1% by weight) of total CE in plasma.

One limitation of this study is that the present informatics approach provides only suggestions, but not conclusions. Based on the suggestions, however, we can plan future experiments to test the suggestions. For example, the suggested uniqueness of CE 22:6 might raise a possibility of a unique metabolic pathway, which might lead us to identify possible receptors or transporters specific to CE 22:6. We conclude that informatics approach can act as a bridge from the present massive and complex data to a simple and focused study in the future.

**Conclusions**

Use of two basic dimension reduction methods (PCA and factor analysis), had advantages in the analysis of CE molecules in the epidemiological study. PCA and factor analysis seemed to suggest the quantity, the food source, fat solubility, and the biological function of CE molecules. Factor analysis following to a factor selection process using BIC and RMSEA was found more practical than PCA in this study. Informatics approach will be useful for analysis of a large and complex lipid dataset generated by mass spectrometry. This study uncovered the uniqueness of CE 22:6,
which remains to be elucidated in the future.

Acknowledgements

This study was financially supported by grants from Integration Research for Agriculture and Interdisciplinary Fields (14538261), and from the Japan Society for the Promotion of Science, KAKENHI (16K15353, 18H03207 and 19K07861).

References

1. Y. Morisawa, Anal. Sci., 2019, 35, 833.
2. Q. Zhang, H. Xu, R. Liu, P. Gao, X. Yang, W. Jin, Y. Zhang, K. Bi, and Q. Li, Anal. Chem., 2019, 91, 3389.
3. Y. Zhang, Y. Liu, J. Wei, S. Xiong, and Z. Zhao, Talanta, 2016, 150, 88.
4. N. Rifai, G. R. Warnick and M.H. Dominiczak, “Handbook of Lipoprotein Testing”, 2002, AACC Press, Washington D. C., U.S.A..
5. E. Warensjö, J. Sundström, L. Lind, and B. Vessby, Am. J. Clin. Nutr., 2006, 84, 442.
6. B. Vessby, A. Aro, E. Skarfors, L. Berglund, I. Salminen, and H. Lithell, Diabetes, 1994, 43, 1353.
7. M. Öhrvall, L. Berglund, I. Salminen, H. lithell, A. Aro, and B. Vessby, Atherosclerosis, 1996, 27, 65.
8. Y. Miura, T. Furukawa, M. Kobayashi, R. Shrestha, R. Takahashi, C. Shimizu, H. Chiba, and S.-P.Hui, Steroids, 2017, 123, 43.
9. Y. Miura, S.-P. Hui, M. Kobayashi, R. Shrestha, T. Hiruma, S. Takeda, H. Fuda, S. Ikegawa, K. Hirano, and H. Chiba, Steroids, 2016, 107, 1.
10. K. Nakamura, S.-P. Hui, S. Ukawa, E. Okad, T. Nakagawa, H. Okabe, Z. Chen, Y. Miura, H. Chiba, A. Tamakoshi, Sleep Med., 2019, 57, 135.
11. K.V. Mardia, J.T. Kent, and J.M. Bibby, “Multivariate analysis”, 1994, ACADEMIC PRESS, San Diego, CA, U. S. A.

12. A Practical Introduction to Factor Analysis: Exploratory Factor Analysis, Compilation prepared by UCLA Institute for Digital Research & Education, https://stats.idre.ucla.edu/spss/seminars/introduction-to-factor-analysis/a-practical-introduction-to-factor-analysis/

13. K.J. Preacher, G. Zhang, C. Kim, and G. Mels, Multivariate Behav. Res., 2013, 48, 28.

14. A.C. Rustan, and C.A. Drevon, “Fatty acids: Structure and properties” in “Encyclopedia of Life Science”, 2005, 1.

15. J.S. Patton, B. Stone, C. Papa, R. Abramowitz, and S.H. Yalkowsky, J. Lipid Res., 1984, 25, 189.

16. E. Ricciotti and G.A. FitzGerald, Arterioscler. Thromb. Vasc. Biol., 2011, 31, 986.

17. R.J.S. Lacombe, R. Chouinard-Watkins, and R. P. Bazinet, Mol. Aspects Med., 2018, 64, 109.

18. Y. Pan, M. J. Scanlon, Y. Owada, Y. Yamamoto, C. J. Porter, and J. A. Nicolazzo, Mol. Pharm., 2015, 12, 4375.

19. C.N. Serhan, Nature, 2014, 510, 92.

20. L.N. Nguyen, D. Ma, G. Shui, P. Wong, A. Cazenave-Gassiot, X. Zhang, M. R. Wenk, E. L. K. Goh, and D.L. Silver, Nature, 2014, 509, 502.

21. B.H. Wong, J. P. Chan, A. Cazenave-Gassiot, R. W. Poh, J. C. Foo, D. L. A. Galam, S. Ghosh, L. N. Nguyen, V. A. Barathi, S.W. Yeo, Chi. D. Luu, and M.R. Wenk, J. Biol. Chem., 2016, 291, 10501.
### Table 1  Average concentrations of CE molecules

| Molecules | Total       | Gender       | Age (years) |
|-----------|-------------|--------------|-------------|
|           | Male (n=545)| Female (n=300)| 30-39 (n=52) | 40-49 (n=88) | 50-59 (n=114) | 60-69 (n=157) | 70-79 (n=134) |
| CE 16:0   | 0.619±0.242 | 0.619±0.351  | 0.620±0.354 | 0.526±0.163 | 0.533±0.222 | 0.582±0.285 | 0.664±0.383 | 0.693±0.252 |
| CE 18:0   | 0.024±0.014 | 0.024±0.015  | 0.025±0.016 | 0.022±0.007 | 0.023±0.011 | 0.025±0.014 | 0.025±0.016 | 0.025±0.014 |
| CE 18:1   | 0.798±0.317 | 0.813±0.46   | 0.786±0.453 | 0.722±0.223 | 0.705±0.287 | 0.773±0.384 | 0.852±0.503 | 0.848±0.324 |
| CE 18:2   | 1.046±0.387 | 1.013±0.567  | 1.074±0.606 | 1.041±0.321 | 1.001±0.41  | 1.07±0.522 | 1.074±0.61 | 1.027±0.426 |
| CE 18:3   | 0.047±0.024 | 0.045±0.027  | 0.049±0.031 | 0.039±0.012 | 0.043±0.018 | 0.047±0.024 | 0.05±0.032 | 0.05±0.028 |
| CE 20:4   | 0.175±0.067 | 0.172±0.096  | 0.177±0.102 | 0.171±0.053 | 0.167±0.07  | 0.169±0.083 | 0.184±0.107 | 0.176±0.069 |
| CE 20:5   | 0.133±0.08  | 0.129±0.083  | 0.135±0.09  | 0.092±0.032 | 0.103±0.05  | 0.12±0.062 | 0.145±0.095 | 0.164±0.087 |
| CE 22:6   | 0.037±0.013 | 0.035±0.019  | 0.038±0.022 | 0.03±0.01   | 0.032±0.013 | 0.037±0.018 | 0.038±0.022 | 0.040±0.013 |

Values are mean±SD (μmol/L)
Table 2  Comparison in indexes of different factor numbers for free fatty acids

| Factor number | 2     | 3     | 4     | 5     |
|---------------|-------|-------|-------|-------|
| RMSEA index   | 0.246 | 0.126 | 0.092 | NA    |
| BIC value     | 355.62| 22.71 | -1.47 | NA    |

RMSEA: the root mean square error of approximation, that is a chi-square-based calculation for each simulation. RMSEA value of between 0.08 and 0.1 indicates a mediocre fit, and below 0.08 indicates a good fit. BIC value: Bayesian information criterion value, that is a maximum-likelihood-based method for model selection. Model with the lowest BIC value is preferred.
Table 3  Comparison in uniqueness of free fatty acids, total fatty acids, and cholesteryl esters

| Acyl structure | Free fatty acids | Total fatty acids | Cholesteryl esters |
|---------------|------------------|-------------------|-------------------|
| 16:0          | 0.054            | 0.037             | 0.204             |
| 18:0          | 0.121            | 0.193             | 0.005             |
| 18:1          | 0.049            | 0.119             | 0.044             |
| 18:2          | 0.005            | 0.005             | 0.296             |
| 18:3          | 0.005            | 0.005             | 0.320             |
| 20:4          | 0.530            | 0.572             | 0.005             |
| 20:5          | 0.509            | 0.107             | 0.005             |
| 22:6          | 0.315            | 0.217             | 0.467             |

Uniqueness of one variable means how its variance to the variable not shared with other variables.

Note that CE 22:6 showed the highest uniqueness value among all CE, whereas CE 20:5 that is another n-3 polyunsaturated CE showed the lowest uniqueness value. In free fatty acids and total fatty acids, acyl 22:6 did not show the highest uniqueness value.
Figure legends

Fig. 1  Principal component analysis. Biplot of first principal component (PC1) and second principal component (PC2). PC1 was considered to reflect size, and PC2 was considered to reflect food source.

Fig. 2  Principal component analysis. Biplot of third principal component (PC3) and forth principal component (PC4). PC3 was considered to reflect fat solubility. What PC4 reflected was not specified.

Fig. 3  Factor analysis. Biplot of factor 1 and factor 2. Factor 1 was considered to reflect size. Factor 2 was considered to reflect food source.

Fig. 4  Factor analysis. Biplot of factor 3 and factor 4. Factor 3 was considered to reflect fat solubility. Factor 4 was considered to reflect inflammation.
Figure 1
Figure 2
Figure 3
Figure 4