Lipid classification of fish oil omega-3 supplements in ethyl ester and triacylglycerols forms by ^{1}H NMR and multivariate analysis

Abstract:

The worldwide advent of concentrated supplements containing omega-3 fatty acids (FA) in the form of triacylglycerols (TAG) or ethyl esters (EE) has increased the interest in developing methods to classify these products. The quality control based on their lipid composition has become necessary since EE bioavailability has been proved to be lower when compared to the TAG. In this preliminary study, eight models based on ^{1}H NMR and supervised discriminant analysis (PLS-DA/OPLS-DA) were applied to classify omega-3 fish oil in TAG or EE forms. The 4.0-4.5 ppm region was selected for modeling since it bracketed spectral features to discriminate TAG and EE. The non-supervised principal component analysis was employed to visually evaluate the distribution of samples and revealed a clear separation of TAG from EE marine oils along PC1. In addition, representative TAG and EE samples were 100% correctly classified using any of the eight supervised models studied. The developed models resulted in high R^2Y ($\geq$ 0.977) and Q^2 ($\geq$ 0.953), and low root mean square error for prediction ($\leq$ 0.009), which demonstrates the high potential of this rapid and straightforward procedure to evaluate the lipid form of supplements and mislabeling.

Keywords: principal component analysis; discriminant analysis; ethyl esters; triacylglycerol; marine oil.

1. Introduction

Omega-3 fatty acids (FA) consumption has several positive effects on human health, being recognized effective against cardiovascular and inflammatory diseases, among others (Nichols et al., 2014). Fish oil supplements containing eicosapentaenoic (EPA) and docosahexaenoic (DHA) are one of the most consumed supplements with a remarkable market worldwide. Natural fish oils generally contain EPA and DHA as triacylglycerols (TAG), while concentrated supplements usually have those FA in ethyl ester (EE) form. The conversion of EE into TAG can be carried out after molecular distillation, but it is very costly and commonly bypassed by manufacturers (Rubio-Rodríguez et al., 2010), which leads to a high volume of commercialized concentrated supplements composed mostly of EE. Nevertheless, incorporation of EPA and DHA into plasma phospholipids has been evidenced to be significantly different, showing a better bioavailability of omega-3 FA in the TAG form when compared to EE (Ghasemifard et al., 2014). For these reasons, it is extremelly important to monitor FA form in fish oil supplements.

The increased demand for fish oil omega-3 supplements requires fast and accurate methods to verify both product quality and label accuracy. Gas chromatography is the most commonly used technique to determine the FA in fish oils (AOCS, 2013). However, previous derivatization of analytes is necessary, long and specific capillary columns are required, and methods are usually time-consuming and laborious.

On the other hand, proton nuclear magnetic resonance (^{1}H NMR) spectrum can be obtained in less than 1 min, a minimal amount of sample is required for experiments due to the deep length and small cross-sectional area of NMR tubes, and small amounts of organic solvents are needed (~500 µL). When considering TAG and EE, the potential of ^{1}H NMR (Lopes et al., 2020) and ^{13}C NMR (Suárez et al., 2010) was already demonstrated for the discrimination of these

compounds in marine oils. Vibrational spectroscopic techniques were also employed for this purpose (Karunathilaka et al., 2020; Killeen et al., 2017). For quantitative analysis of EPA and DHA, Wu and He (2014) and Wu *et al.* (2014) have already described methods employing NMR and chemometrics, but only TAG were approached. To the best of our knowledge, NMR associated with a multivariate analysis has never been used for the supervised classification of fish oils according to their lipid form (TAG, EE). Methods developed by spectroscopic techniques associated with multivariate calibration are simple, fast, and follow the green analytical chemistry principles (Gałuszka et al., 2013), due to the straightforward analysis and little waste generated during experiments.

Overall, this work focused in developing fast and straightforward discriminating methods for the classification of fish oil supplements according to the available form of FA (TAG or EE) employing ^{1}H NMR spectroscopy and supervised discriminant analysis (partial least square discriminant analysis, PLS-DA/orthogonal partial least square discriminant analysis, OPLS-DA). The method of the present research can be applied to quality control analysis of fish oils in industries and regulatory agencies, and it would also be very useful to verify the label accuracy of these supplements. 1H NMR Spectra can be acquired in a few minutes without any complex sample preparation, and the use of discriminant analysis permit to determine group membership of samples with minimal error using a group of predictors, which can be performed by non-experienced NMR analysts.

2. Materials and Methods

2.1. Samples, Test Mixtures, and Reagents

Eighteen different dietary omega-3 fish oil supplements commercialized in capsules were acquired from local and online markets (Brazil, Canada, Spain, and the USA). These samples were used in the calibration and cross-validation steps of the PLS-DA model after classification in TAG or EE by referenced chromatographic techniques (thin layer chromatography - TLC and gas chromatography – GC-MS). Twelve samples were TAG, and six samples were EE.

Additionally, a set of 18 mixtures of pure TAG or EE fish oil samples were prepared to evaluate the model performance by mixing three different pure TAG (or different pure EE). Equal amounts of fish oil were added to glass tubes, mixed by gentle agitation, and stored at -80 °C in an inert atmosphere until analyses.

Deuterated chloroform and 2',7'-dichlorofluorescein were acquired from Sigma-Aldrich (St. Louis, MO, USA). Hexane, chloroform, ethanol, diethyl ether, and glacial acetic acid were purchased from Panreac Química S.A. (Madrid, Spain).

2.2. Analytical Methods

Five mg of fish oil were dissolved in 500 μL of deuterated chloroform and inserted in a 5 mm tube for ^{1}H NMR analysis. Experiments were performed with spinning on a Bruker Avance III HD spectrometer operating at 500 MHz, and spectra were acquired by the Topspin 3.2 software from Bruker Biospin. No sample preprocessing was initially applied to ^{1}H NMR spectra.

The experimental parameters for ^{1} H NMR experiments were as follows: data acquisition, 3.28 s; relaxation delay, 1.0 s; pre-scan delay, 10.00 μs; total data points collected, 65536; spectral width, 20.0 ppm; scans, 16 (no dummy scans); pulse angle, 30 °; temperature, 25 °C. The chemical shifts were reported in ppm relative to the solvent residual peak (chloroform 7.28 ppm).

Sample preparation and analysis by TLC and GC-MS carried out for fish oil unequivocally classification were performed as described previously (Amorim et al., 2020).

2.4 Multivariate Data Analysis

Multivariate analysis was performed in SIMCA P+ version 12.0.1 - Umetrics. Principal component analysis (PCA) was used as an unsupervised exploratory tool to visually evaluate the distribution of all fish oil samples containing FA as TAG or EE. Data were mean-centered before analysis, and three principal components (PC) were selected. Then, PLS-DA and OPLS-DA (Bylesjö et al., 2006; Trygg and Wold, 2002) were used to predict the sample class (TAG or EE). Multivariate analysis was performed using the spectral range of 4.0-4.5 ppm (1672 data points), selected based on ^{1}H NMR spectra interpretation. PLS-DA and OPLS-DA models were initially developed using raw ^{1}H NMR spectra data. Then, some pretreatments, such as the standard normal variate (SNV) and the Savitzky-Golay's smoothing algorithm were applied, individually or simultaneously, using the SIMCA P+ software package.

The developed models were validated internally using the leave-n-out (Shao, 1993) cross-validation method (18 samples) and externally using a series of TAG and EE test mixtures (18 samples), as described in section 2.1. The first step in modeling was recoding the categorical variables (TAG and EE) into continuous numerical variables. A one (1) indicated a sample belonging to TAG class, and a zero (0) indicated a sample not belonging to this class (i.e., belonging to EE class). For each unknown test sample, the model predicted a y-value, which should ideally be close to either 1 or 0. The decision rule established for fish oil classification was: samples with predicted $y > 0.5$ and a deviation <0.5 belong to TAG class; samples with predicted $y < 0.5$ and a deviation <0.5 belong to EE class, and samples with a deviation > 0.5 can not be properly

classified. PLS-DA and OPLS-DA models were evaluated considering the root-mean square-error of prediction (RMSEP), R^2X, R^2Y, Q^2, p-value from CV-ANOVA (Eriksson et al., 2008), sensitivity, false negatives, specificity, false positives, Younden's index, and Matthews correlation coefficient.

3. Results and Discussion

3.1. Characteristics of ^{1}H NMR fish oil spectra

Fig. 1-a, and Fig. 1-b show representative ^{1}H NMR spectra for fish oil in TAG and EE forms, respectively. The assignation of ressonances was based on Sacchi et al., (2008), Spyros and Dais, (2013), and Alexandri et al., (2017). Spectra evidence similarities and differences, due to both the FA composition of samples and the lipidic form of FA (TAG and EE). The main resonances commonly found in fish oil occur at 0.85 ppm for methyl groups of saturated, n-6 and n-9 FA (signal A); 0.95 ppm for methyl groups of n-3 FA (signal B); 1.25 ppm for methylenic hydrogens of acyl groups (signal C); 1.62 ppm for methylenic hydrogens in β-position of the carbonyl group (signal D); from 2.02 to 2.09 ppm for methylenic hydrogens related to α single double bond (signal E); from 2.2 to 2.5 ppm for methylenic hydrogens α-position of the carbonyl group (signal F); 2.85 ppm for bis-allylic hydrogens (signal G) and at approximately 5.3 ppm corresponding to olefinic hydrogens bearing the acyl group (signal H).

Signals ranging from 4.0 to 4.5 ppm are useful to differentiate TAG and EE forms in fish oil samples. This fingerprint region was employed for subsequent PCA and discriminant analysis. Resonances H_1 (4.15-4.35 ppm, doublet of doblet) and I (5.3 ppm, multiplet) are due to glyceryl group hydrogens at positions *sn*-1, *sn*-3 (H_1), and *sn*-2 (I), characteristics of TAG, as illustrated in

Fig. 1-a, while resonance H_2 (4.12 ppm, multiplet) is characteristic for the terminal methylenic hydrogen of EE forms, as illustrated in Fig. 1-b.

3.2. Principal component analysis

This unsupervised exploratory tool was useful to check for anomalies and also observe the distribution of samples. PC1 and PC2 described 76.59 % and 9.11 % of the variance in the spectral data set, respectively. Fig. 2 shows the scores plot (A) and the loadings plot (B) of PCA. Evaluation of Fig. 2-A revealed a clear separation of TAG and EE fish oils along PC1. TAG samples (red circles) clustered in the positive PC1, whereas EE samples (blue squares) grouped in the negative scores of the same PC, which is in accordance with the loadings showed in Fig. 2-B, where it is possible to see a negatively loaded band at approximately 4.15 ppm (characteristic of EE) and a positively loaded band at 4.3-4.35 ppm (characteristic of TAG) in blue color (PC1 loadings).

TAG samples were grouped closer because they presented very similar omega-3 contents. All samples contained 540 mg EPA and 360 mg DHA in the daily serving size (3000 mg), according to the nutrition facts, which was also verified in a previous study (Amorim et al., 2020). EE samples, on the other hand, had very different omega 3 contents. According to the nutrition facts, supplements could provide from 120 to 1008 mg EPA and from 240 to 756 mg DHA in a daily portion, which could vary from 600 to 2800 mg FO.

Moreover, there was no evidence of outliers. Some samples were observed outside the hotteling confidence limit (e.g., sample 13, Fig 2-A), but they presented a similar NMR spectra when compared with the other samples from the same group. Then, all samples from PCA were considered in the modeling.

158 3.3 Classification of samples by PLS-DA and OPLS-DA

159 To classify samples as TAG or EE, PLS-DA and OPLS-DA models were built by using
160 the raw ^{1}H NMR data initially, as described in section 2.3 (models M1 and M2, respectively).
161 Then, PLS-DA and OPLS-DA models were studied using some data pre-processement (SNV in
162 models M3 and M4, Savitzky Golay in models M5 and M6, and SNV+ Savitzky Golay in models
163 M7 and M8).

164 Calibration models were developed using 18 commercial samples, previously analyzed
165 by TLC and GC-MS. The reference TLC method permitted to clearly distinguish TAG from EE
166 samples based on the difference in the retention factor, as depicted in Fig 3 (*i.e.*, TAG retention
167 factor = 0.6 and EE retention factor = 0.8). Also, analyses by GC-MS corroborated previous
168 classifications, since TAG and EE presented different m/z spectra and different elution times
169 (Amorim et al., 2020).

170 Table 1 shows Y values predicted for each fish oil sample, considering the 8 models
171 evaluated (M1-M8). It can be observed that calculated values were very close to 1 for all TAG
172 samples (Samples 1-12 and 19-30), whereas calculated values were very close to 0 for EE samples
173 (Samples 13-18 and 31-36). Also, it can be noticed that PLS-DA and OPLS-DA models provided
174 very similar results. The score plots of PLS-DA and OPLS-DA, as well the PCA models, were
175 supplied in the supplementary material.

176 The performance of the studied models was measured in terms of sensitivity and
177 specificity and considering some classification metrics (R^2X, R^2Y, Q^2, RMSEP, and p-values from
178 CV-ANOVA), reported in Table 2. The sensitivity of the model represents the proportion of test
179 samples belonging to the class that was correctly classified, and the specificity represents the
180 proportion of test samples that did not belong to the class that was correctly identified

(Karunathilaka et al., 2019). All models studied (M1-M8) have shown 100% of both sensitivity and specificity since all TAG and EE samples were correctly classified (Table 2). False negatives and false positives were not identified. Younden's index and Matthews correlation coefficient were equal to 1. Moreover, all models were significant since p-values < 0.05 were calculated (Eriksson et al., 2008).

Even considering the models developed with ^{1}H NMR raw data (M1 and M2), good results were obtained (100% of test samples were classified correctly, $R^2Y \geq 0.977$, $Q^2 \geq 0.953$, RMSEP ≤ 0.009092). However, the SNV preprocessing treatment or the synergistic effect of SNV and the smoothing Savitzky-Golay algorithm provided the best prediction results, with the lowest RMSEP (≤ 0.003893) and the highest R^2Y (≥ 0.986) and Q^2 (≥ 0.968), as shown in Table 2.

Overall, the current study was successful in demonstrating the potential of ^{1}H NMR associated with PLS-DA/OPLS-DA in the supervised classification of TAG and EE pure fish oils. This approach is faster and less laborious than classical TLC or gas chromatography method and requires a small amount of solvents to acquire NMR spectra. As perspectives for this preliminary study, improved models could be evaluated with more complex samples, including TAG and EE mixtures, and also considering quantitative analysis. The suitability of portable instrumentation for this same approach is also interesting to be studied. Besides, the use of ^{1}H NMR is advantageous when compared to ^{13}C NMR or two dimensional NMR techniques, due to its higher throughput. Other NMR techniques associated with multivariate modeling can also be evaluated for fish oil classification, since previous papers already reported the use of high-resolution multinuclear (^{1}H, ^{13}C, ^{31}P) and multidimensional NMR spectroscopy for fatty acids determination in fish oils, but only signal integration was considered (Bratu et al., 2012; Dais et al., 2015; Igarashi et al., 2002; Williamson and Hatzakis, 2017).

Conclusions

The use of ^{1}H NMR spectra associated with PLS-DA or OPLS-DA was successful in discriminating fish oil samples according to the lipid form of omega-3 fatty acids (TAG or EE). 100% of test samples were correctly classified using the developed supervised models. The present method is extremely fast because ^{1}H NMR spectra can be obtained in less than 1 min. Furthermore, a small amount of sample (5 mg) and solvent (500 µL) is required, being a non-destructive analysis that follows green chemistry principles. Multivariate analysis is of special interest in the context of food authentication, and the association of ^{1}H NMR with PLS-DA offers a simple, fast, and precise analysis, without requiring highly experienced analysts for data interpretation. This procedure has great potential to be used in quality control analysis by industry and regulatory agencies and to evaluate supplements label accuracy.

References

Alexandri, E., Ahmed, R., Siddiqui, H., Choudhary, M.I., Tsiafoulis, C.G., Gerothanassis, I.P., 2017. High resolution NMR spectroscopy as a structural and analytical tool for unsaturated lipids in solution. Molecules 22, 1–71. https://doi.org/10.3390/molecules22101663

Amorim, T.L., De La Fuente, M.A., De Oliveira, M.A.L., Gómez-Cortés, P., 2020. Evaluation of Delivery Form of Eicosapentaenoic and Docosahexaenoic Acids During Quality Control of Fish Oil Supplements. Brazilian J. Anal. Chem. 7, 20–30. https://doi.org/10.30744/brjac.2179-3425.AR-19-2020

AOCS, 2013. Official Method Ce 1i-07. Determination of saturated, cis-monounsaturated, and cis-polyunsaturated fatty acids in marine and other oils containing long chain polyunsaturated fatty acids (PUFAs) by capillary GLC.

Bratu, A., Mihalache, M., Hanganu, A., Chira, N.-A., Todasca, M.-C., Rosca, S., 2012. Quantitative determination of fatty acids from fish oils using GC-MS method and 1H-NMR spectroscopy. UPB Sci. Bull. Ser. B Chem. Mater. Sci. 75, 23–32.

Bylesjö, M., Rantalainen, M., Cloarec, O., Nicholson, J.K., Holmes, E., Trygg, J., 2006. OPLS discriminant analysis: Combining the strengths of PLS-DA and SIMCA classification. J. Chemom. 20, 341–351. https://doi.org/10.1002/cem.1006

Dais, P., Misiak, M., Hatzakis, E., 2015. Analysis of marine dietary supplements using NMR spectroscopy. Anal. Methods 7, 5226–5238. https://doi.org/10.1039/c5ay00794a

Eriksson, L., Trygg, J., Wold, S., 2008. CV-ANOVA for significance testing of PLS and OPLS® models. J. Chemom. 22, 594–600. https://doi.org/10.1002/cem.1187

Gałuszka, A., Migaszewski, Z., Namieśnik, J., 2013. The 12 principles of green analytical

chemistry and the SIGNIFICANCE mnemonic of green analytical practices. TrAC - Trends Anal. Chem. 50, 78–84. https://doi.org/10.1016/j.trac.2013.04.010

Ghasemifard, S., Turchini, G.M., Sinclair, A.J., 2014. Omega-3 long chain fatty acid "bioavailability": A review of evidence and methodological considerations. Prog. Lipid Res. 56, 92–108. https://doi.org/10.1016/j.plipres.2014.09.001

Igarashi, T., Marit Aursand, R., Sacchi, L.P., Michio Nonaka, S.W., 2002. Determination of docosahexaenoic acid and n-3 fatty acids in refined fish oils by 1H-NMR spectroscopy: IUPAC interlaboratory study. J. AOAC Int. 85, 1341–1354.

Karunathilaka, S.R., Hwan, S., Mossoba, M.M., Jean, B., Brückner, L., Ellsworth, Z., Srigley, C.T., 2019. Rapid classification and quantification of marine oil omega-3 supplements using ATR-FTIR , FT-NIR and chemometrics. J. Food Compos. Anal. 77, 9–19. https://doi.org/10.1016/j.jfca.2018.12.009

Karunathilaka, S.R., Yakes, B.J., Choi, S.H., Brückner, L., Mossoba, M.M., 2020. Comparison of the Performance of Partial Least Squares and Support Vector Regressions for Predicting Fatty Acids and Fatty Acid Classes in Marine Oil Dietary Supplements by Using Vibrational Spectroscopic Data. J. Food Prot. 83, 881–889.

Killeen, D.P., Marshall, S.N., Burgess, E.J. et al, 2017. Raman spectroscopy of fish oil capsules: polyunsaturated fatty acid quantitation plus detection of ethyl esters and oxidation. J. Agric. Food Chem. 65, 3551–3558. https://doi.org/https://doi.org/10.1021/acs.jafc.7b00099

Lopes, T.I.B., Pereira, E.S., Freitas, D. dos S., Oliveira, S.L., Alcantara, G.B., 2020. Spectral profiles of commercial omega-3 supplements: an exploratory analysis by ATR-FTIR and 1H NMR. J. Food Sci. Technol. 57, 1251–1257. https://doi.org/10.1007/s13197-019-04157-y

261 Nichols, P.D., McManus, A., Krail, K., Sinclair, A.J., Miller, M., 2014. Recent advances in
262 omega-3: Health benefits, Sources, Products and bioavailability. Nutrients 6, 3727–3733.
263 https://doi.org/10.3390/nu6093727
264 Rubio-Rodríguez, N., Beltrán, S., Jaime, I., de Diego, S.M., Sanz, M.T., Carballido, J.R., 2010.
265 Production of omega-3 polyunsaturated fatty acid concentrates: A review. Innov. Food Sci.
266 Emerg. Technol. 11, 1–12. https://doi.org/10.1016/j.ifset.2009.10.006
267 Sacchi, R., Savarese, M., Falcigno, L., Giudicianni, I., Paolillo, L., 2008. Proton NMR of Fish
268 Oils and Lipids, in: Webb, G.A. (Ed.), Modern Magnetic Resonance. Springer, Dordrecht.
269 https://doi.org/https://doi.org/10.1007/1-4020-3910-7_105
270 Shao, J., 1993. Linear model selection by cross-validation. J. Am. Stat. Assoc. 88, 486–494.
271 https://doi.org/10.1080/01621459.1993.10476299
272 Spyros, A., Dais, P., 2013. NMR Spectroscopy in Food Analysis. RSC Publishing, Cambridge.
273 Suárez, E.R., Mugford, P.F., Rolle, A.J., Burton, I.W., Walter, J.A., Kralovec, J.A., 2010. 13C-
274 NMR regioisomeric analysis of EPA and DHA in fish oil derived triacylglycerol
275 concentrates. JAOCS, J. Am. Oil Chem. Soc. 87, 1425–1433.
276 https://doi.org/10.1007/s11746-010-1638-2
277 Trygg, J., Wold, S., 2002. Orthogonal projections to latent structures (O-PLS). J. Chemom. 16,
278 119–128. https://doi.org/10.1002/cem.695
279 Wu, D., He, Y., 2014. Potential of spectroscopic techniques and chemometric analysis for rapid
280 measurement of docosahexaenoic acid and eicosapentaenoic acid in algal oil. Food
281 Chemistry. 158, 93–100. http://dx.doi.org/10.1016/j.foodchem.2014.02.109
282 Wu, D., Chen, X., Cao, F., Sun, D., He, Y., Jiang, Y., 2014. Comparison of Infrared
283 Spectroscopy and Nuclear Magnetic Resonance Techniques in Tandem with Multivariable

Selection for Rapid Determination of ω-3 Polyunsaturated Fatty Acids in Fish Oil. Food Bioprocess Technol. 7, 1555–1569. https://doi.org/10.1007/s11947-013-1147-z

Williamson, K., Hatzakis, E., 2017. NMR Spectroscopy as a Robust Tool for the Rapid Evaluation of the Lipid Profile of Fish Oil Supplements. J. Vis. Exp. 1–14. https://doi.org/10.3791/55547

290 **Figure Captions:**

291

292 Fig 1: Raw ^{1}H NMR spectra of a) a natural fish oil supplement containing fatty acids as
293 triacylglycerols and b) a concentrated fish oil supplement containing fatty acids as ethyl esters.

294

295 Fig 2: Scores plot for unsupervised principal component analysis (A) and loadings plot (B).

296

297 Fig 3: Thin-layer chromatography of 18 commercial fish oil containing fatty acids as
298 triacylglycerols (retention factor = 0.6) and ethyl esters (retention factor = 0.8).

299

Supplementary material:

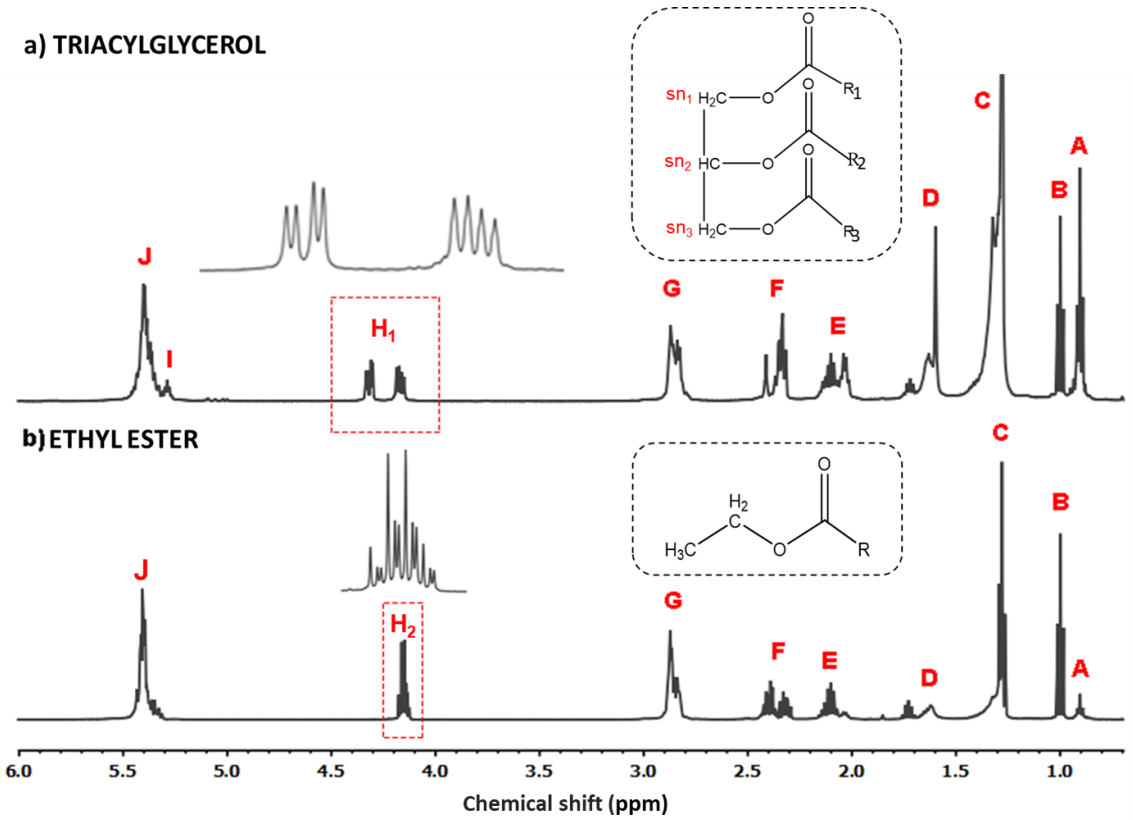
Fig 1: Score plots of PCA, PLS-DA and OPLS-DA models using raw NMR data

Fig 2: Score plots of PCA, PLS-DA and OPLS-DA models using SNV pre-treatment

Fig 3: Score plots of PCA, PLS-DA and OPLS-DA models using Savitzky Golay pre-treatment

Fig 4: Score plots of PCA, PLS-DA and OPLS-DA models using SNV and Savitzky Golay pre-treatments

Fig 1:



Signal	Proton
A	-C**H**$_3$
B	-C**H**$_3$ (n-3)
C	-(C**H**$_2$)$_n$-
D	-OCO-CH$_2$-C**H**$_2$-
E	-CH=CH-C**H**$_2$-
F	-OCO-C**H**$_2$-
G	-CH=CH-C**H**$_2$-CH=CH-
H$_1$ (TAG)	-C**H**$_2$-OCOR (sn$_1$ e sn$_3$)
H$_2$ (EE)	CH$_3$-C**H**$_2$-OCOR
I (TAG)	-C**H**-OCOR (sn$_2$)
J	-C**H**=C**H**-

Fig 2:

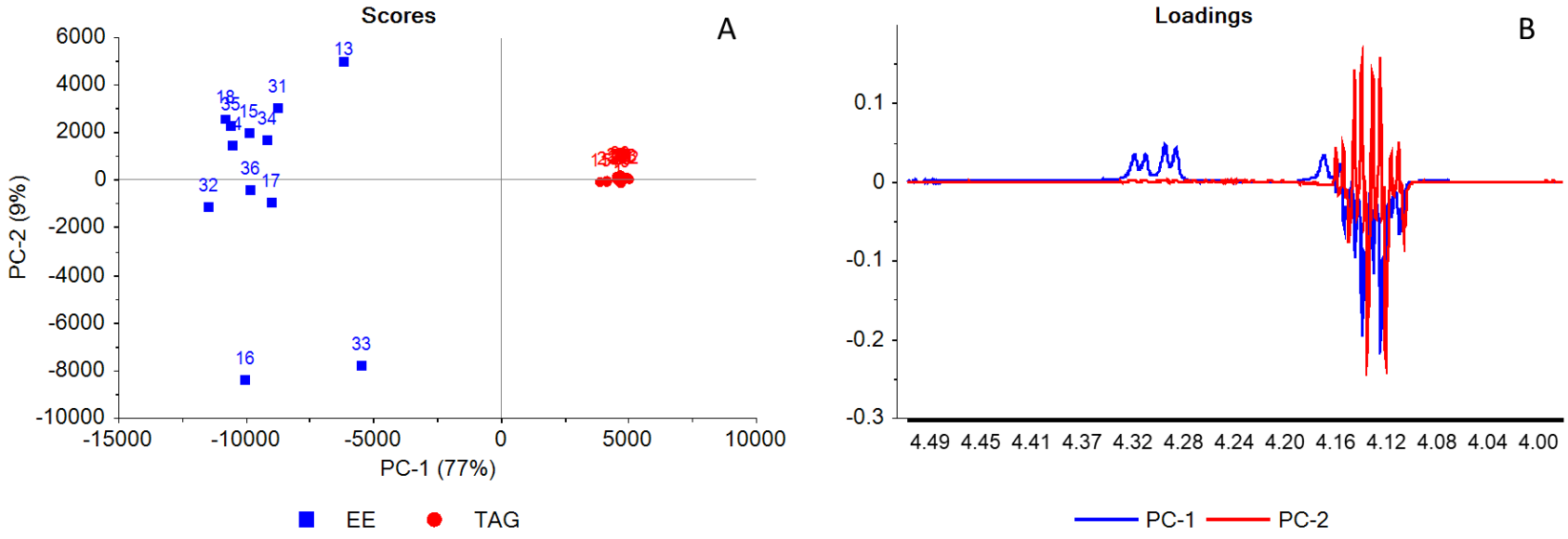
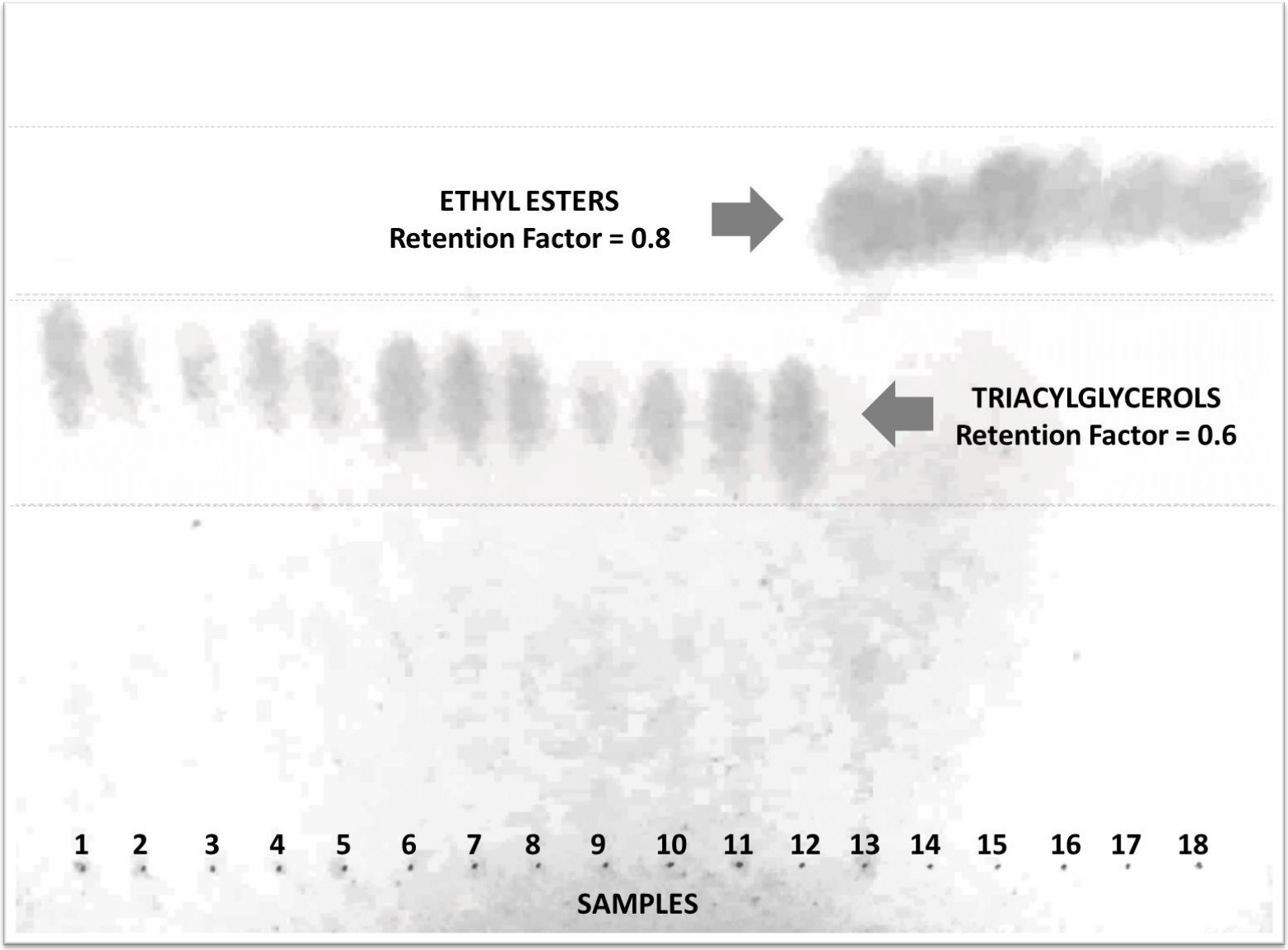
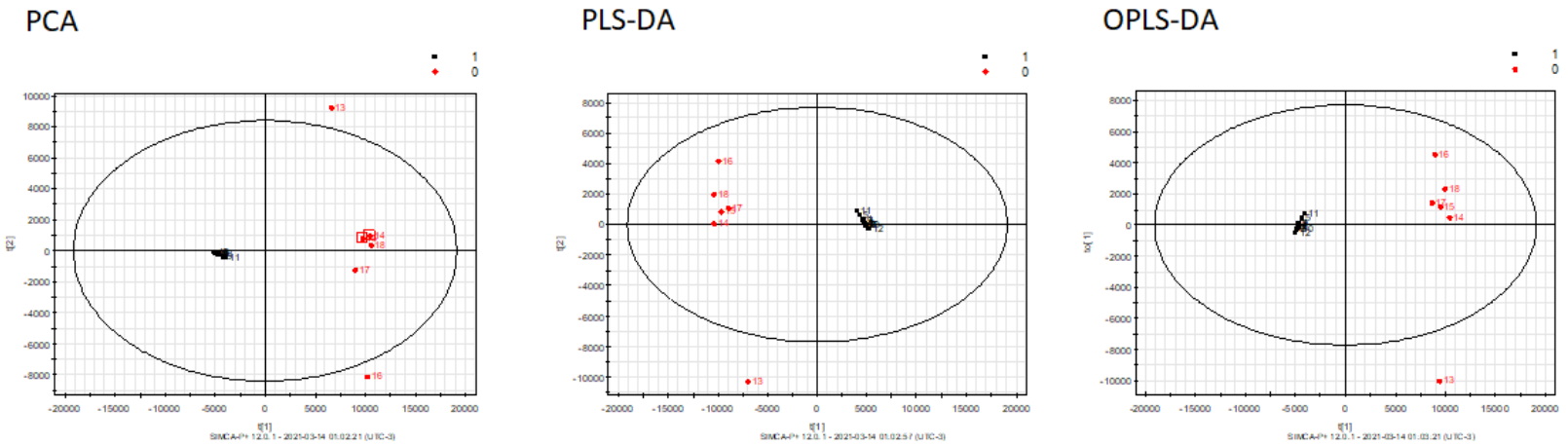

Scores
A
6000
4000
2000
0
-2000
-4000
-6000
-8000
-10000
PC-2 (9%)
-15000
-10000
-5000
0
5000
10000
PC-1 (77%)
13
31
18
35
15
34
4
36
32
17
16
33
EE
TAG
Loadings
B
0.1
0
-0.1
-0.2
-0.3
4.49 4.45 4.41 4.37 4.32 4.28 4.24 4.20 4.16 4.12 4.08 4.04 4.00
PC-1
PC-2

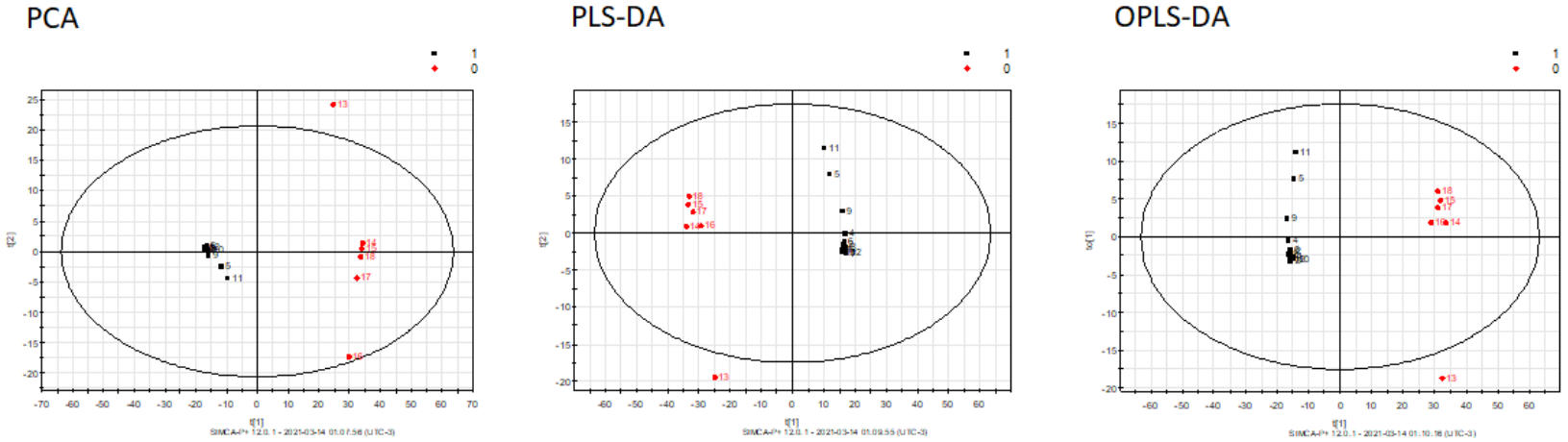
Fig 3:


ETHYL ESTERS
Retention Factor = 0.8
TRIACYLGLYCEROLS
Retention Factor = 0.6
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
SAMPLES

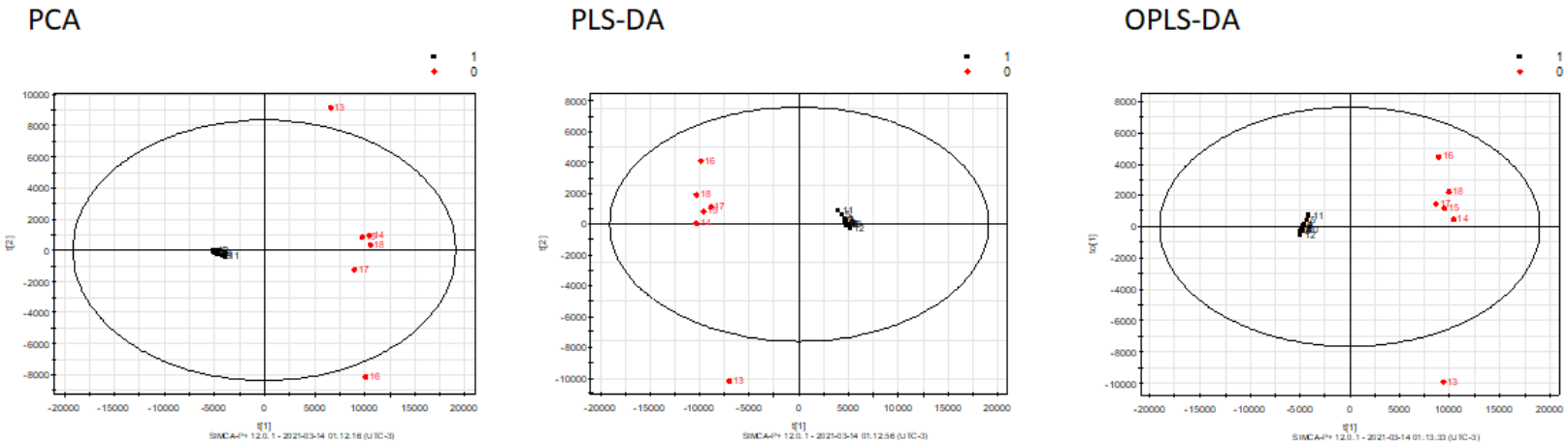
Supplementary material – Fig 1:



Supplementary material – Fig 2:



Supplementary material – Fig 3:



Supplementary material – Fig 4:

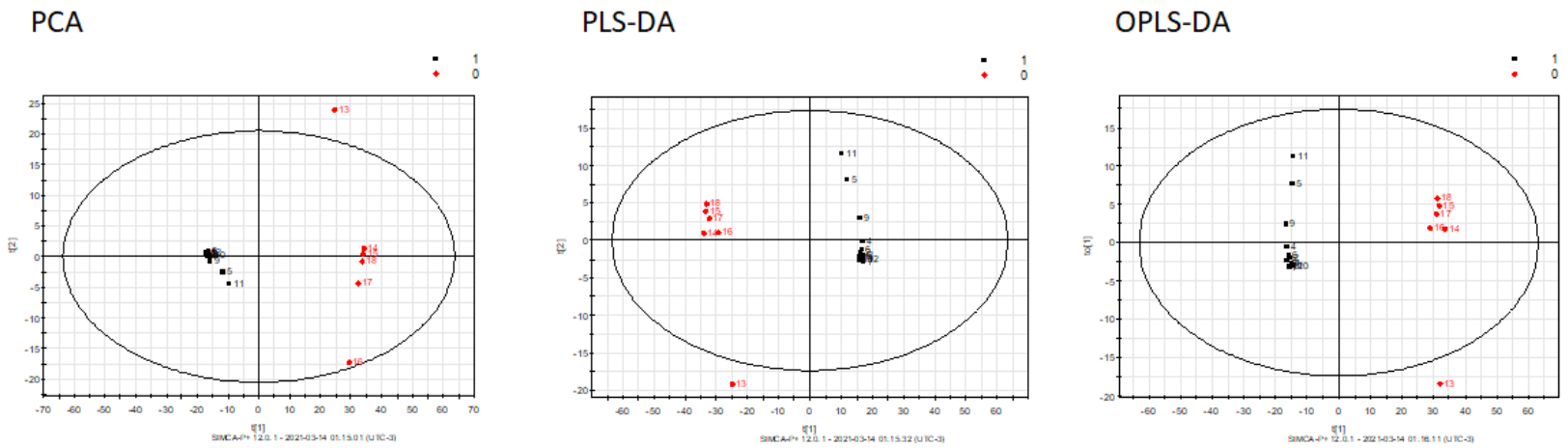


Table 1: Values predicted by partial least square discriminant analysis (PLS-DA) and orthogonal partial least squares discriminant analysis (OPLS-DA) considering ^{1}H NMR data. 18 commercial fish oil supplements were used for calibration and cross-validation. 18 test mixtures of pure triacylglycerol (TAG) and ethyl ester (EE) were employed for prediction. Variables: 1672.

Sample	Class	M1	M2	M3	M4	M5	M6	M7	M8
1	1	0.920728	0.920728	1.003880	1.003880	0.920692	0.920692	1.003850	1.003850
2	1	0.988301	0.988301	1.009900	1.009900	0.988276	0.988276	1.009910	1.009910
3	1	1.014260	1.014260	1.017160	1.017160	1.014240	1.014240	1.017180	1.017180
4	1	0.954589	0.954589	1.018850	1.018850	0.954566	0.954566	1.018870	1.018870
5	1	0.952465	0.952465	0.927191	0.927191	0.952410	0.952410	0.927155	0.927155
6	1	0.977477	0.977477	1.008260	1.008260	0.977457	0.977457	1.008290	1.008290
7	1	1.028460	1.028460	1.025100	1.025100	1.028440	1.028440	1.025130	1.025130
8	1	1.050920	1.050920	1.031010	1.031010	1.050900	1.050900	1.031050	1.031050
9	1	0.985482	0.985482	0.997448	0.997447	0.985446	0.985446	0.997446	0.997446
10	1	1.015640	1.015640	0.996364	0.996364	1.015590	1.015590	0.996333	0.996333
11	1	0.835497	0.835497	0.891450	0.891450	0.835464	0.835464	0.891479	0.891479
12	1	1.101960	1.101960	1.026710	1.026710	1.101940	1.101940	1.026750	1.026750
13	0	0.167281	0.167281	0.153732	0.153732	0.167271	0.167271	0.153385	0.153385
14	0	-0.068963	-0.068963	-0.059838	-0.059837	-0.069191	-0.069191	-0.059926	-0.059926
15	0	-0.026829	-0.026829	-0.044918	-0.044918	-0.027046	-0.027046	-0.044979	-0.044979
16	0	-0.012476	-0.012476	0.071331	0.071331	-0.011745	-0.011745	0.071992	0.071992
17	0	0.049226	0.049226	-0.025950	-0.025950	0.048790	0.048790	-0.026271	-0.026271
18	0	-0.057225	-0.057225	-0.033203	-0.033203	-0.057249	-0.057249	-0.032960	-0.032960
19	1	0.952315	0.952315	1.031380	1.031380	0.952300	0.952300	1.031420	1.031420
20	1	0.979996	0.979996	1.025440	1.025440	0.979964	0.979964	1.025440	1.025440
21	1	0.848386	0.848386	0.926525	0.926525	0.848341	0.848341	0.926459	0.926459
22	1	0.962761	0.962761	1.033920	1.033920	0.962748	0.962748	1.033970	1.033970
23	1	1.035950	1.035950	1.046300	1.046300	1.035930	1.035930	1.046360	1.046360
24	1	0.921948	0.921948	1.015110	1.015110	0.921928	0.921928	1.015130	1.015130
25	1	0.981954	0.981954	1.030560	1.030550	0.981936	0.981936	1.030600	1.030600
26	1	0.922431	0.922430	0.999472	0.999472	0.922404	0.922404	0.999480	0.999480
27	1	0.904375	0.904375	1.005460	1.005460	0.904357	0.904357	1.005490	1.005490
28	1	0.981430	0.981430	1.016530	1.016530	0.981401	0.981401	1.016530	1.016530
29	1	0.949256	0.949256	1.003030	1.003030	0.949224	0.949224	1.003020	1.003020
30	1	0.970858	0.970858	1.018440	1.018440	0.970841	0.970841	1.018470	1.018470
31	0	0.042654	0.042654	-0.027117	-0.027117	0.042188	0.042188	-0.027301	-0.027301
32	0	-0.103477	-0.103477	-0.051289	-0.051289	-0.103864	-0.103864	-0.051428	-0.051428
33	0	0.305957	0.305957	0.226710	0.226710	0.304396	0.304396	0.225464	0.225464
34	0	0.034751	0.034751	-0.029204	-0.029204	0.035029	0.035030	-0.028688	-0.028688
35	0	-0.050645	-0.050645	-0.040317	-0.040317	-0.050464	-0.050464	-0.039968	-0.039968
36	0	0.007548	0.007548	-0.026189	-0.026189	0.008047	0.008047	-0.025549	-0.025549

Class: 1 for TAG and 0 for EE. M1: PLS-DA/raw data; M2: OPLS-DA/raw data; M3: PLS-DA/SNV; M4: OPLS-DA/SNV; M5: PLS-DA/Savitzky Golay; M6: OPLS-DA/Savitzky Golay; M7: PLS-DA/SNV+Savitzky Golay; M8: OPLS-DA/SNV+Savitzky Golay.

Table 2: Parameters for the evaluation of models using partial least square discriminant analysis (PLS-DA) and orthogonal partial least squares discriminant analysis (OPLS-DA) considering ^{1}H NMR data.

Model	A	N	R^2X(cum)	R^2Y(cum)	Q^2(cum)	p-values CV-ANOVA	RMSEP	Sensitivity (%)	Specificity (%)
M1	2	18	0.875	0.977	0.972	4.63E-09	0.009092	100	100
M2	1+1+0	18	0.875	0.977	0.953	1.63E-08	0.009092	100	100
M3	2	18	0.913	0.986	0.977	1.12E-09	0.003893	100	100
M4	1+1+0	18	0.913	0.986	0.968	1.35E-09	0.003893	100	100
M5	2	18	0.877	0.977	0.972	4.44E-09	0.009044	100	100
M6	1+1+0	18	0.877	0.977	0.954	1.54E-08	0.009044	100	100
M7	2	18	0.914	0.986	0.977	1.07E-09	0.003859	100	100
M8	1+1+0	18	0.914	0.986	0.968	1.28E-09	0.003859	100	100

A: latent variables; N: observations; RMSEP: root-mean square-error for prediction.