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RESEARCH PAPER

Authentication of fish oil (omega-3) supplements using class-oriented chemometrics and comprehensive two-dimensional gas chromatography coupled to mass spectrometry

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Abstract

Food supplement authentication is an important concern worldwide due to the ascending consumption related to health benefits and its lack of effective regulation in underdeveloped countries, making it a target of fraudulent activities. In this context, this study evaluated fish oil supplements by comprehensive two-dimensional gas chromatography coupled to mass spectrometry (GC×GC–MS) to obtain fingerprints, which were used to build predictive models for automated authentication of the most popular products sold in Brazil. The authentication process relied on a one-class classifier model using data-driven soft independent modeling of class analogy (DD-SIMCA). The output of the model was a binary classifier: certified IFOS fish oils and non-certified ones — regardless of the source of adulteration. The compositional analysis showed a significant variation in the samples, which validated the need for reliable statistical models. The DD-SIMCA algorithm is still incipient in GC×GC studies, but it proved to be an excellent tool for authenticity purposes, achieving a chemometric model with a sensitivity of 100%, specificity of 98.6%, and accuracy of 99.0% for fish oil authentication. Finally, orthogonalized partial least square discriminant analysis (OPLS-DA) was used to identify the features that distinguished the groups, which ascertained the results of the DD-SIMCA model that IFOS-certified oils are positively correlated to omega-3 fatty acids, including eicosapentaenoic acid (EPA, C20:5 n-3) and docosahexaenoic acid (DHA, C22:6 n-3).

Keywords Artificial intelligence · Authentication · Data science · Foodomics · GCxGC-MS · Machine learning

Introduction

Food supplements are an important source of bioactive substances, which can be sold as capsules or liquid dosage forms to complement the regular diet [1–4]. In 2016, the food supplement industry contributed with over 120 billion dollars to the US economy [5].

Many food supplements are commercially available, and fish oil is the most popular product worldwide [6, 7], probably due to several reports showcasing important health benefits [8–10]. Such benefits are related to the occurrence of polyunsaturated fatty acids, wherein the most important are the omega-3 (n-3) constituents [11, 12]. Fish oil is rich in eicosapentaenoic acid (EPA, C20:5 n-3) and docosahexaenoic acid (DHA, C22:6 n-3), which are different omega-3 fatty acids exclusively found in marine products [11–13]. Other fatty acids are found in seeds and plant oils such as alpha-linolenic acid (C18:3 n-3), linoleic acid (C18:2 n-6), arachidonic acid (C20:3 n-6), oleic acid (C18:1 n-9), and nervonic acid (C24:1 n-9) [11, 12].

The increasing demand for fish oil and the lack of effective regulation in some countries, such as underdeveloped countries, are a potential scenario for fraudulent practices, like mislabeling and adulteration with lower quality ingredients [1, 3, 13, 14]. For this reason, the assessment of fish oil quality has raised concerns among consumers and regulatory

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agencies worldwide [13, 14]. This awareness has led to the implementation of the international fish oil standards (IFOS), which is the oldest and most popular certification by Nutrasource, a Canadian company [15]. The IFOS-certified products meet the levels of omega-3 fatty acid described on the labels and are tested to check for possible oxidation and contaminants found in fish, including dioxins, furans, mercury, and lead [15]. Consequently, the IFOS-certified products are typically more expensive than the non-certified analogs, which raises concerns about the safety and quality of the latter products. Hence, quality assurance protocols are desperately needed to monitor fish oil supplements.

Multiple analytical platforms are needed to assess the inorganic and organic constituents of fish oils. However, this study focuses on the analysis of the fatty acids found in such products, which are the main ingredients of this supplement. Several studies were reported evaluating fish oil quality using different analytical techniques [14], such as vibrational spectroscopy [16–20], nuclear magnetic resonance [19, 21, 22], liquid chromatography (LC) [23–25], and gas chromatography (GC) [13, 26–28]. More specifically, an interesting technique for the analysis of the individual fatty acid isomers is comprehensive two-dimensional gas chromatography (GC×GC) [29].

Comprehensive two-dimensional gas chromatography is a multidimensional separation technique that explores two sequential GC stages to improve the overall peak capacity of the composite system [30–33], being considered an ideal platform for untargeted analysis in foodomic investigations [34]. Important reports of fish oil analysis using GC×GC include analysis of dioxins in fish oil [35], profiling of fatty acid methyl esters (FAME) in menhaden fish oil [36], and occurrence of persistent organic pollutants in fish oils [37]. However, to the best of our knowledge, there are no studies using GC×GC to study fish oil certification.

Fundamental studies with product certification and adulteration are challenging because the quality assurance and nature of potential adulterants are highly dependent on the country. Usually, underdeveloped countries are more susceptible to adulterations, which challenges multiclass classification. For instance, discriminant analysis using partial least squares (PLS-DA) may be unsuccessful if many impurities and adulterants are used, as the intra-class variation may become too large, jeopardizing the performance of the chemometric model. Moreover, if an adulterant was not used in the training dataset but is detected in test data, then the sample might be misclassified by the PLS-DA model. In this context, one-class chemometric models are particularly useful, as the certification model relies solely on the characteristics of the desired product, regardless of the impurities or adulterants [38, 39].

More specifically, data-driven soft independent modeling of class analogy (DD-SIMCA) may be explored for one-class classifiers, being an ideal solution for food supplement (e.g., fish oil) authentication resulting in a binary decision. In other words, the model output informs the analyst if the sample is authentic or not — regardless of the reason why the adulterated exhibited a different chromatographic profile. If discriminant analysis was used, the number of classes being modeled likely would increase with the number of adulterants being addressed by the investigation [40, 41]. To accomplish this goal, authentic samples are graphically assigned within a statistically relevant acceptance area, while the model rejects non-authentic samples, as described in the "DD-SIMCA" section.

In this study, we evaluated an alternative method for fish oil authentication according to the IFOS certification. The thirty-two selected brands were the most popular fish oil supplements sold through e-commerce in Brazil. The experiments were carefully designed for the use of one-class classifiers using DD-SIMCA chemometric modeling [38, 39]. A pixel-based data analysis approach was employed using the GC×GC chromatograms as chemical fingerprints (i.e., untargeted analysis) for the classification model [42–45], followed by orthogonalized partial least squares discriminant analysis (OPLS-DA) with the sole purpose of generating a loading tensor to validate the findings of the DD-SIMCA. Finally, important figures of merit for the DD-SIMCA classification model were determined during method development, including sensitivity, specificity, and accuracy [46]. It is hoped that this report will highlight the potential application of one-class classifiers to authentication studies with chemometrics in the field of GC×GC.

Materials and methods

Materials and samples

The chemicals used during the derivatization process were sodium methoxide, formic acid, dichloromethane, and sodium sulfate (Sigma-Aldrich, St, Louis, MO, USA). The 2-mL tubes were purchased from Eppendorf (São Paulo, SP, Brazil). Methanol and methylene chloride were purchased from Synth (Diadema, São Paulo, Brazil), and sodium sulfate was purchased from CAQ (Diadema, São Paulo, Brazil). A set of 2-mL screw-top glass vials with PFTE/PDMS septum was purchased from Nova Analítica (São Paulo, São Paulo, Brazil). The samples consisted of 32 brands of commercial fish oil supplement capsules purchased from e-commerce (Table 1). The experiments were performed with true replicates ($N = 3$). Peak identification was confirmed using the Supelco 37 Component FAME Mix (Sigma-Aldrich), alongside confirmation with mass spectral library search (Wiley and NIST) and selected references [36, 47].

Table 1 Description of the fish oil supplements evaluated in this study

Sample ID	EPA (mg)	DHA (mg)	Batch	Certification	Ingredients
A1	840	521	51,666	IFOS	Fish oil and alpha tocopherol (vitamin E)
A2	600	400	206,373	IFOS	Fish oil
A3	1080	720	21,040,232	IFOS	Fish oil and alpha tocopherol (vitamin E)
A4	867	512	61,252,020	IFOS	Fish oil and alpha tocopherol (vitamin E)
A5	720	480	63,102,113	IFOS	Fish oil and alpha tocopherol (vitamin E)
A6	840	521	55,611	IFOS	Fish oil and alpha tocopherol (vitamin E)
A7	1048	424	2,120,103	IFOS	Fish oil and alpha tocopherol (vitamin E)
B1	160	100	25,087	-	Fish oil, linseed oil, and borage seed oil
B2	540	360	P210581	-	Fish oil
B3	360	240	3,201,085	GOED	Fish oil
B4	540	360	159,400,220	-	Fish oil
B5	360	240	24,183	-	Fish oil
B6	360	240	6416	-	Fish oil
B7	360	240	2,010,542	-	Fish oil
B8	530	360	2145	-	Fish oil
B9	400	200	72,021	-	Fish oil
B10	540	360	2146	-	Fish oil
B11	540	360	P210386	-	Fish oil
B12	540	360	A213671	-	Fish oil
B13	270	180	15,024/2	-	Fish oil
B14	270	180	2186	-	Fish oil
B15	540	360	2,113,003	-	Fish oil
B16	540	360	1754	-	Fish oil
B17	540	360	P200522	-	Fish oil
B18	360	240	61	-	Fish oil
B19	540	360	210,413	-	Fish oil
B20	540	360	491,212	-	Fish oil
B21	540	360	2669	-	Fish oil
B22	540	360	P210003	-	Fish oil
B23	390	244	12,602	-	Fish oil
B24	270	180	40,621	-	Fish oil
B25	540	360	A214025	-	Fish oil

Derivatization method

The procedure was adapted from Bogusz et al. [48]. An aliquot of 200 mg of fish oil sample was weighted in a 2 mL tube. An aliquot of 200 µL of saturated sodium methoxide in methanol was added. The reaction proceeded at 70ºC for 5 min using a MULTI REAX vortex mixer (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany). Next, an aliquot of 100 µL of formic acid solution (0.5 mol L^{-1}) was added to neutralize the basic catalyst. To remove all remaining water, sodium sulfate was added to the tube. Then, 1 mL of methylene chloride was added and vortexed for 15 s. The tubes containing the samples were centrifuged (ROTANTA 460R, Hettich, Beverly, MA, USA) for 5 min at 9056 × units of gravity (g). Finally, 600 µL of the supernatant was collected and transferred to a 2-mL screw-top glass for GC×GC analysis.

Instrumentation

Comprehensive two-dimensional gas chromatography coupled with mass spectrometry (GC×GC–MS) was used to obtain the FAME profile of the 32 fish oil supplements. The system consisted of a TRACE 1300 GC coupled to an ISQ single transmission quadrupole mass spectrometer (ThermoFisher Scientific, Waltham, MA, USA). A TriPlus RSH autosampler (ThermoFisher Scientific) was used to inject 1 µL of the liquid sample with a 60:1 split ratio at 280 °C. The non-polar × mid-polar column configuration consisted of a first 20 m × 0.18 mm-ID (0.18 µm film thickness) SLB-1 ms column (100% poly(dimethylsiloxane)) (Supelco, Bellefonte, PA, USA) and a second 2.5 m × 0.25 mm-ID (0.25 µm film thickness) MEGA-17 column (poly(diphenyl-dimethylsiloxane) with

50% diphenylsiloxane monomer incorporation) — adapted from [49].

The flow modulation was performed using the reverse fill/flush configuration [50] employing three-port and four-port SilFlow GC splitters (Trajan Scientific and Medical, Melbourne, Australia) [51, 52]. A 50-µL sampling loop comprising a 23 cm × 0.53 mm-ID MXT deactivated guard column (Restek Corporation) and a bleed capillary of 2.5 m × 0.10 mm-ID were employed for flow modulation [53]. A three-way miniature diaphragm isolation valve (The Lee Company, Westbrook, CT, USA) was used to actuate the auxiliary gas during flow modulation. A modulation period of 4 s with a flush period of 250 ms was used. For flow splitting, an unpurged 3-port SilFlow GC splitter (Trajan Scientific) and two 5.0 m × 0.18 mm-ID and 5.0 m × 0.32 mm-ID fused silica capillaries (Restek Corporation) were used for passive division of the ^{2}D flow. The 0.18 mm-ID and 0.32 mm-ID capillaries were used as the transfer line to the MS and FID, respectively.

The oven temperature was programmed from 160 °C to 290 °C at 3 °C min^{-1}. The transfer line and ion source temperatures were set at 280 °C and 250 °C, respectively. Helium was used as carrier and auxiliary gas at 0.5 mL min^{-1} and 25 mL min^{-1}, respectively. EI was performed at 70 eV. Full MS (centroid mode) was used for data acquisition (25.60 Hz) with a spectral range of 50 to 400 u at nominal mass resolution. Blank runs were performed every 5 injections for quality assurance.

Xcalibur (ThermoFisher Scientific) software was used for instrument control and data acquisition. ChromSpace (SepSolve Analytical, Waterloo, ON, Canada) was employed to control the flow-modulator and synchronize the GC run. GC Image (GC Image, LLC, Lincoln, NE, USA) was used for qualitative analysis using the template matching feature for batch processing. The "*.RAW" Xcalibur files were converted to "*.CDF" ANDI/netCDF format using the file converter plug-in. Chemometrics was performed on MATLAB R2021a (MathWorks, Natick, MA, USA) environment. The netCDF files were imported to MATLAB to generate three-way data tensors. DD-SIMCA was performed using a modified algorithm from Pomerantsev et al. [38, 39].

Data preprocessing

A pixel-based approach was selected for multivariate data processing [54, 55]. The interested reader is directed elsewhere for more information on handling non-integer acquisition rates [56]. The GC×GC chromatograms were imported to MATLAB as three order tensors (i.e., data cube) (^{1}D by ^{2}D by m/z). The augmented matrices were prepared by unfolding them to a matrix (^{1}D by ^{2}D × *m/z*) for one-class analysis using DD-SIMCA [38, 39]. The peaks from the blank runs were removed from the dataset prior to chemometrics. Next, intensities of all ions in the corresponding mass spectra (i.e., belonging to the same scan) were summed up to create the total ion chromatograms (TIC), which were organized into a matrix (samples by TIC). Finally, chromatograms were normalized, Pareto-scaled, and mean-centered before the modeling step. The interested reader is directed elsewhere for more information on the preprocessing steps [57]

DD-SIMCA

Classification is a statistical problem that aims to answer to which class an unknown object belongs, based on a model that contains objects whose categories are well-established [41]. A category or class is a group of objects that can be defined by common properties shared among all its members. Therefore, classification methods will differ from each other on how to statistically estimate the class limits in a multivariate space.

Pomerantsev et al. [38] proposed in 2008 an algorithm that uses distances based on leverage and residual variance from principal component analysis (PCA) to assort unknown objects. The method named DD-SIMCA is based on SIMCA, which is a multiclass well-established classification algorithm proposed by Wold [58]. In DD-SIMCA, score distance (SD) and orthogonal distance (OD) standardized by a chi-squared (χ^2) distribution are used to describe a function that delimits a single-class acceptance area with a significance level (e.g., 95%). To calculate these limits, scaling factors (h_0 and v_0) and the number of degrees of freedom (N_h and N_v) are needed. These parameters can be estimated from a training dataset (X). The first step consists in the decomposition of X ($I \times J$), as in PCA:

$$X = TP^t + E \tag{1}$$

where T ($I \times A$) is the score matrix, with A being related to the used number of principal components (PC), P ($J \times A$) is the loadings matrix, and E ($I \times J$) is the residuals matrix. In the second step, the calculated scores (T) and loadings (P) enable the extraction of h_i and v_i for score and orthogonal distances, respectively:

$$h_i = t_i t\left(T^t T\right)^{-1} t_i = \sum\nolimits_{a=1}^{A} \frac{t_{ia}^2}{\lambda_a} \tag{2}$$

where λ_a, $a = 1, \ldots, A$ are the diagonal elements of the matrix T^tT:

$$v_i = \sum\nolimits_{j=1}^{J} e_{ij}^2 \tag{3}$$

When both OD and OS are calculated, the total distance c can be calculated for each sample:

$$c = N_h \frac{h}{h_0} + N_v \frac{v}{v_0} \propto \chi^2 \left(N_h + N_v \right) \tag{4}$$

where N_h, h_0, N_v, and v_0 are unknown at first but are estimated by a data-driven approach [38, 59]. Thus, based on a c_{crit} value, we can develop the acceptance area for a given value α [59]. So, from now on any, new sample will be considered authentic, or otherwise, it will be classified as unauthentic — regardless of the features responsible for such differentiation:

$$c \leq c_{crit} = \chi^{-2} \left(\alpha - 1, N_h + N_v \right) \tag{5}$$

This binary decision rule is what delimits objects considered authentic and non-authentic. This characteristic is what differentiates DD-SIMCA from discriminant analysis, such as PLS-DA. In DD-SIMCA, only one well-known class of authentic objects (X, target class) is necessary to allocate news samples as being authentic (or not). Conversely, PLS-DA intrinsically needs predefined classes to do the classification task, which makes it difficult to classify an object that does not belong to any of the pre-established specific classes. In summary, a PLS-based discriminant model will classify a new sample only if this sample is a member of one of the predefined classes, which makes such methods not the most suitable for authentication investigations [41].

OPLS-DA

In this study, OPLS-DA modeling was performed only to obtain the loading array to evaluate the features and ascertain the results obtained with DD-SIMCA [60–62].

Results and discussion

Sample discussion

Fish oil supplement may be considered a complex sample due to the occurrence of many critical pairs of positional isomers of the polyunsaturated fatty acid methyl esters. The successful use of GC×GC was reported to improve the chromatographic resolution of such challenging peak clusters [36, 47]. In addition to the enhanced separation, qualitative analysis also benefited from the ordered and structured GC×GC chromatograms, which evidenced the elution patterns of the FAME peaks. For example, using a non-polar × mid-polar column configuration, it was possible to establish elution regions for the peaks of saturated, mono-, di-, tri-, tetra-, penta-, and hexa-unsaturated FAMEs. This pattern alongside the use of authentic standards enabled the identification of the peaks detected in the GC×GC chromatograms. Interestingly, the number of detected peaks in the fish oil supplements seemed to exhibit fewer peaks than those found in biological samples, like fresh herring oil [47]. Furthermore, the current method was capable of distinguishing between triglycerides and free fatty acids that composed the fish oil supplements — as the evaluated products did not specify the type of lipids used in the formulation. Since transesterification was used for analyte derivatization, it can be stated that the FAMEs originated from triglyceride-like structures due to the nature of the base-catalyzed reaction. However, such reaction does not methylate free fatty acids, so it was possible to detect the free fatty acids as well. While this condition allowed profiling the composition of triglyceride-based fatty acids and the actual free fatty acids, it was observed some tailing in the ^{2}D for the hydrogen-bonding analytes. However, such tailing did not cause any peak overlap in the GC×GC chromatograms. For most samples, the relative abundance of free fatty acids was found between 0 and 2%. Conversely, the relative concentration was 23 to 69% for the samples A6, A7, B3, and B20.

The average composition ($N = 3$) of the 10 most representative samples is shown using a heatmap plot (Fig. 1). Interestingly, there were no clear patterns among the samples, as the fish oil supplements seemed to be composed of triglycerides and free fatty acids arbitrarily with respect to the IFOS certification. In addition, it was seen that the intra-group compositional variation was significant, as highlighted by the hierarchical cluster analysis (HCA) (Fig. 1), which supports the use of a one-class classifier model like DD-SIMCA. The assigned classes 0 (red) and 1 (green) in Fig. 1 represent the samples with and without IFOS certification, respectively.

Noteworthy, the most important fatty acids in fish oil supplements are C20:5 n-3 (EPA) and C22:6 n-3 (DHA). Correlation analysis of the composition of all the samples (Fig. 2) shows that the peak intensities of EPA and DHA are negatively correlated within the samples evaluated in this study. In other words, when the concentration of DHA is high in a particular brand of fish oil, there is less amount of EPA in the same product. Conversely, positive correlations were observed between DHA with shorter chain fatty acids, like C16 and C18, while the same was not be seen for EPA (Fig. 2).

The chemometric approach selected herein was a pixel-based approach for one-class classification using DD-SIMCA. It must be stated that the DD-SIMCA algorithm is suitable for both peak table and pixel-based multivariate data analysis. Particularly, this pixel-based approach was interested in evaluating how the DD-SIMCA algorithm handled chromatograms (Fig. 3) with peak intensities that varied over a wide dynamic range, including column overloading — as highlighted in Fig. 4.

Fig. 1 Hierarchical clustering heatmaps of the fatty acid composition of representative fish oil supplements. The peak areas were normalized and autoscaled. The distance measured was Euclidean. The clustering method used was Ward. The dendrogram illustrates the large intra-class variation among the two evaluated classes, namely, IFOS-certified supplements (class 0, red) and IFOS-non-certified (class 1, green). Abbreviations: free fatty acids (FA), fatty acid methyl esters (FAME)

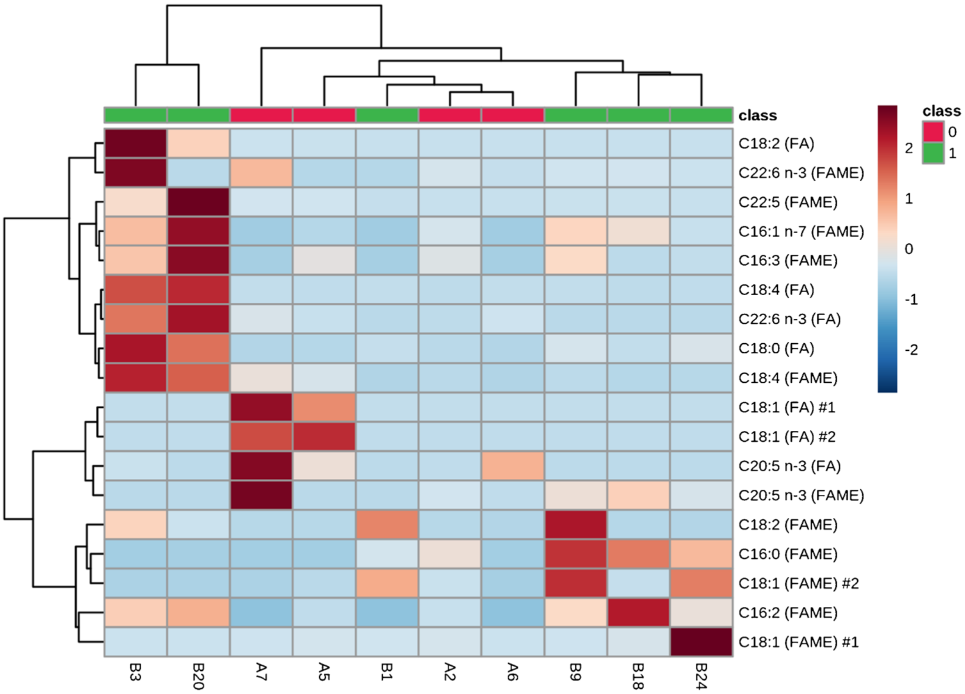
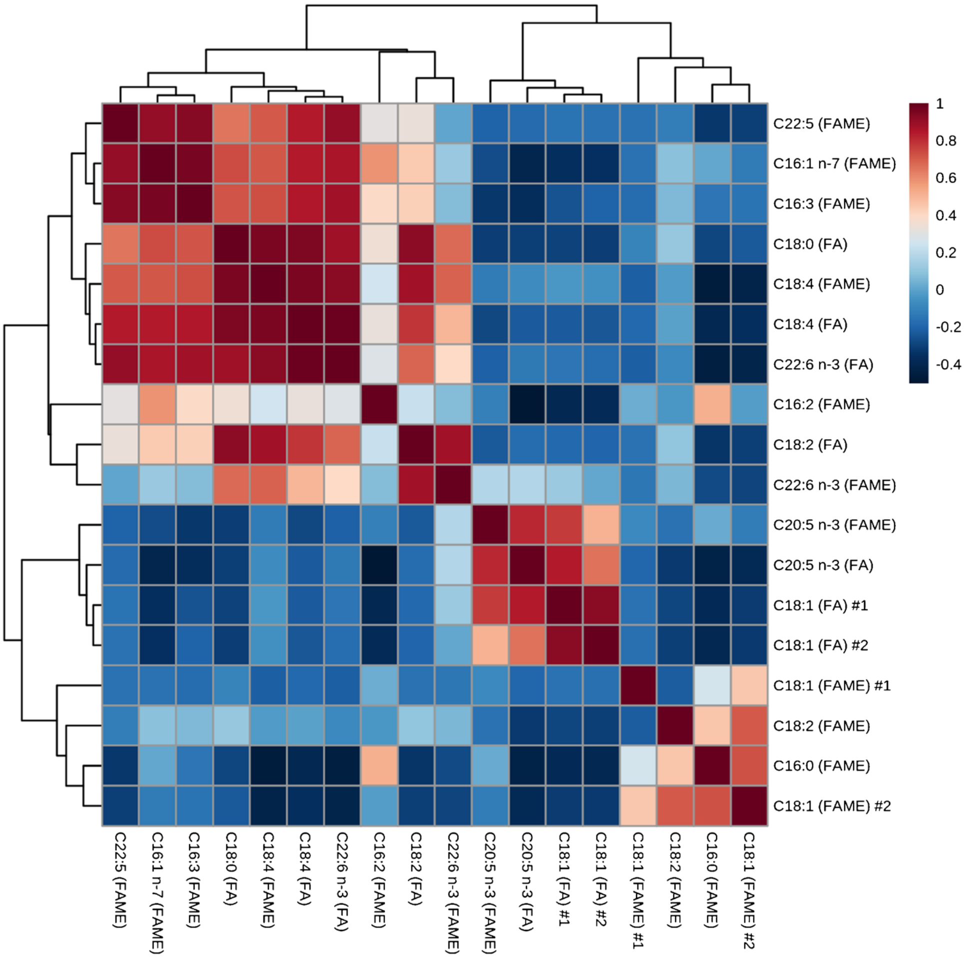


Fig. 2 Correlation heatmaps of the fatty acid composition of fish oil supplements. The data input was the peak areas of the free fatty acids (FA) and the fatty acid methyl esters (FAME). The distance measured was Pearson *r*. The cell color indicates the correlation coefficient between the two selected variables (or features). Caption: positive correlation values (red), negative correlation values (blue)



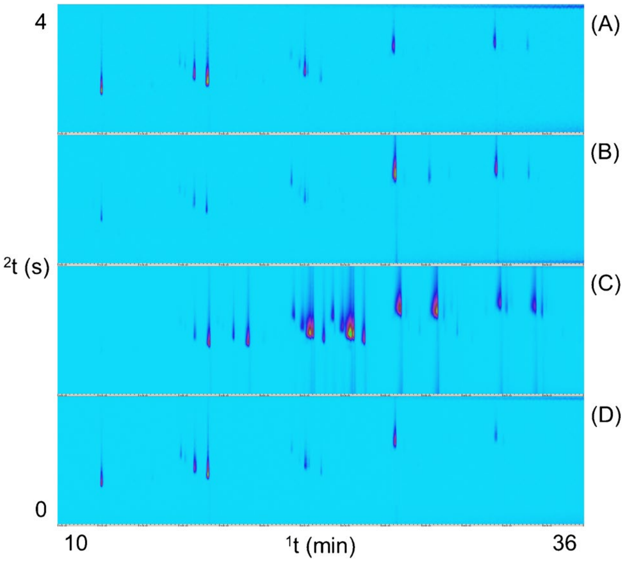


Fig. 3 Total ion GC×GC chromatograms of fatty acid methyl esters (FAME) from fish oil supplements, namely, samples A2 (**A**), A5 (**B**), B3 (**C**), and B18 (**D**)

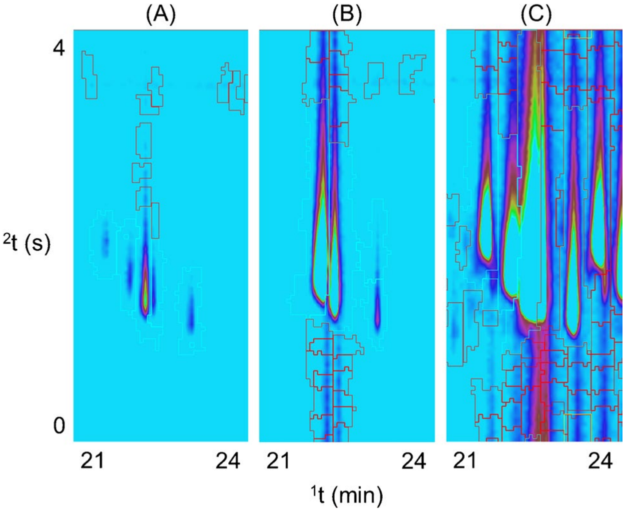


Fig. 4 Expanded total ion chromatogram (TIC) obtained by GC×GC–MS of fish oil supplements. The peaks of the C18:1 isomers exhibited a broad range of intensities in the evaluated fish oil supplements yielding no column overloading (**A**), column overloading (**B**), and excessive column overloading (**C**). As a consequence, the automated peak integration resulted in split peaks (multiple apexes/features for the same compound). This finding motivated the evaluation of pixel-based multivariate data processing to handle the wide dynamic range problem

This specific broad dynamic range problem is already known to be challenging for peak table processing, as overloaded peaks generally cause peak splitting during integration, resulting in a single feature being described with two peak apexes [63].

DD-SIMCA

The first and most important step in creating one-class models is to establish a well-describe class — named target class — in this case, samples with IFOS certificate.

Next, the model was tested against non-certified samples to estimate the figures of merit to evaluate this proof of concept.

All supplements were used for modeling and evaluation, totalizing 7 certified and 25 non-certified samples. The 31 and 72 chromatograms were used for calibration and external validation of the one-class model, respectively. Each chromatogram exhibited 48,123 variables (i.e., pixels). To build the model, the calibration data were normalized and Pareto-scaled. Chi-squared-type acceptance areas were determined, and the number of PCs of the model was set to five the leave-one-out cross-validation method. Outlier significance was set to 0.01, and type I error (α) was calculated as approximately 0.04. The classic estimation method [64] was chosen for the final model (Fig. 5).

The one-class model was mathematically successful as there were no extreme or outliers found in the training dataset (green markers, Fig. 6), corroborating the expected value of 4% type I error (reference value). In the external validation step (blue markers, Fig. 6), only one sample was misclassified, resulting in a type 2 error of 1.4%, which is three times less than the 4.3% calculated for this data set [38, 65]. Important figures of merit like sensitivity (Eq. 6), specificity (Eq. 7), and accuracy (Eq. 8) were calculated for this model by considering the occurrence of true positive

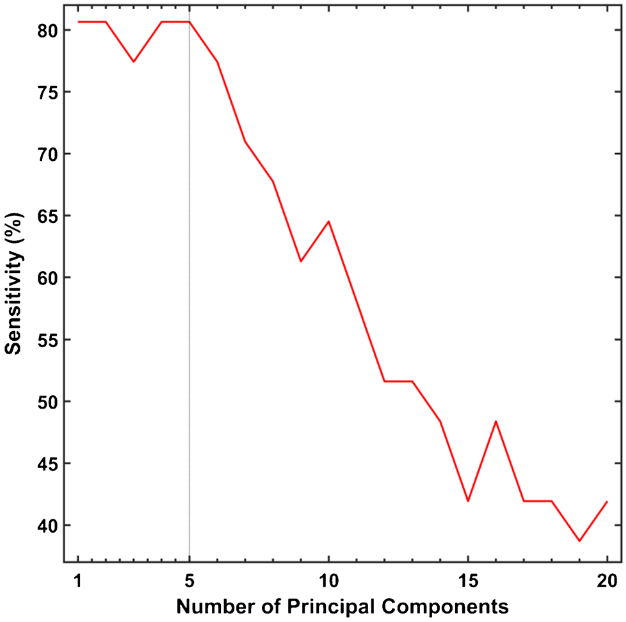


Fig. 5 Leave-one-out cross-validation sensitivity values according to the number of principal components (PCs)

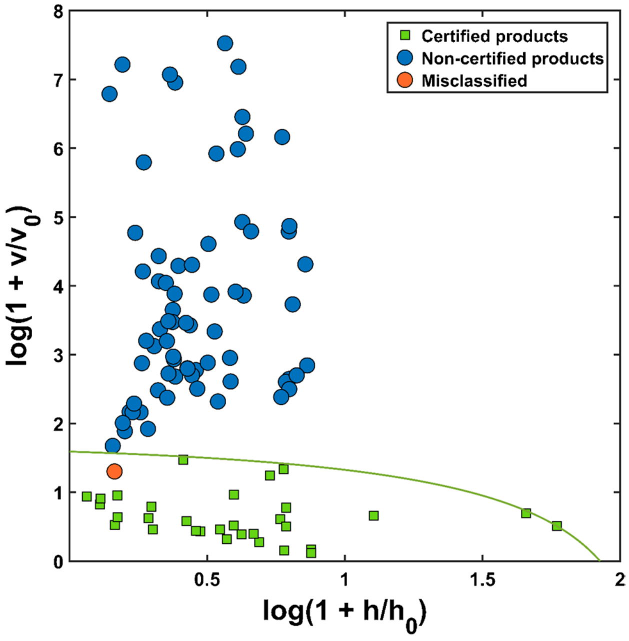


Fig. 6 Acceptance log-chart for IFOS-certified (green squares) and IFOS-non-certified (blue circles) fish oil supplements obtained using DD-SIMCA (one-class classifier). Thirty-two samples were evaluated using true replicates ($N=3$). One replicate of sample B1 was misclassified (orange circle)

(TP), true negative (TN), false positive (FP), and false negative (FN) [46]. This model achieved a sensitivity value of 100%, specificity of 98.6%, and accuracy of 99.0%, which suggests an excellent performance for fish oil authentication, due to a single misclassification. However, when assessing which sample was mismatched, only the second replicate of sample B1 was found. Accordingly, no further actions were necessary.

$$Sensitivity = \frac{TP}{TP + FN} \times 100 \tag{6}$$

$$Specificity = \frac{TN}{TN + FP} \times 100 \tag{7}$$

$$Accuracy = \frac{TP + TN}{TP + TN + FP + FN} \times 100 \tag{8}$$

Remarkably, despite the significant intra-class compositional variation and occurrence of non-linear chromatography (i.e., overloaded peaks in some samples), the one-class DD-SIMCA model was performed adequately for fish oil authentication. This pixel-based chemometric protocol is an interesting alternative for routine studies as all steps involved in data processing can be readily automated and parameters are not subjective. For instance, this pixel-based approach successfully bypassed the need for peak integration, which may require analyst intervention to address split peaks (i.e., one feature and two apexes).

The output differences between DD-SIMCA and PLS-DA must be highlighted to showcase the benefits of one-class classifiers for authentication studies. It is clear from Fig. 6 that the non-certified samples are comprised by more than one class of external objects, above and below log $(1+\frac{v}{v_0})$. This means that in terms of multiclass classification (e.g., PLS-DA), more than one class (at least 2) of non-certified samples was needed to allocate all possibilities of the accredited samples. Furthermore, even if it was beforehand possible to prepare such sub-classes, a discrimination model will likely provide a wrong answer to the authenticity question, once any new object must be attributed to one of the predefined classes. The reason for this limitation is that the analyst must know exactly which adulterants or ingredients differ in the formulations to estimate the number of classes. This procedure is important for multiclass models to minimize the intra-class variation.

Loadings assessment using OPLS-DA

Lastly, the features were evaluated to ascertain the chemical validity of the DD-SIMCA model. Considering that DD-SIMCA does not report loadings arrays, OPLS-DA was used to generate results as similar as possible to the DD-SIMCA model. The interested reader should keep in mind that the classification model used a linear combination of the original pixels to perform the one-class predictions. In other words, the entire chromatogram is used as an input for chemometric modeling, instead of only a handful of "markers".

OPLS-DA was applied to determine which variables are responsible for distinguishing between certified and uncertified samples. Training and validation sets consisted of both IFOS certified and uncertified samples, which were randomly divided to contain 75 and 25% of the samples of each class in those sets respectively, i.e., 23 certified and 54 uncertified chromatograms in the training set and 8 certified and 18 uncertified chromatograms in the validation set. Three latent variables (LV) were selected based on the lowest values of venetian-blind cross-validation average errors. It was found that the first latent variable (LV) was responsible for the discrimination between IFOS-certified and uncertified samples. The certified samples were found in the negative region and the latter in the positive region of the scores plot. Accordingly, the same is valid for the loadings (Fig. 7), since negative loadings are related to the certified samples, whereas the positive loadings are related to the uncertified ones. The peaks in the negative region are related to peaks of C20:5 n-3 (EPA) and C22:6 n-3 (DHA). Conversely, peaks in the positive region are related to other fatty acids (C16 and C18), which was consistent with the

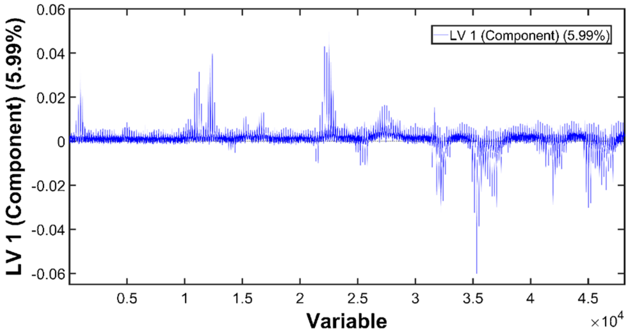


Fig. 7 Loading graph obtained by OPLS-DA of the first latent variable to support the findings obtained with DD-SIMCA. This plot showed that the model used the peaks of EPA and DHA to authenticate the IFOS-certified samples, among other variables

compositional analysis reported in Fig. 2. Interestingly, compositional analysis can lead to misunderstandings in the interpretation of the loadings, as EPA and DHA are not correlated to each other, but might be correlated to C16 and C18 fatty acids (Fig. 2). However, it is important to keep in mind that loadings are coefficients of the linear combination of the variables from which the principal components were constructed. Therefore, higher loading values do not necessarily mean higher peak intensities (or areas for the peak table approach), but rather more importance to the chemometric model.

Conclusion

The proposed method combining GC×GC–MS and one-class classifiers was shown to be interesting alternative for automated authentication of fish oil supplements. The chromatographic method was able to distinguish between the types of lipids that that composed the fish oil supplements, like free fatty acids and triglycerides. Also, it was found that EPA and DHA were negatively correlated within the evaluated samples. In other words, it was found that when DHA is present with higher peak areas, less amount of EPA was found in the same product. In terms of authentication, the chemometric model was considered successful with only one replicate misclassified. Therefore, the final DD-SIMCA model presented a type 2 error of 1.4%, 100% of sensitivity, 98.6% of specificity, and 99.0% of accuracy. Lastly, loadings obtained by OPLS-DA confirmed the findings of DD-SIMCA, wherein the peaks of C20:5 n-3 (EPA) and C22:6 n-3 (DHA) were important to build the chemometric model used for fish oil authentication. However, this task is not to be taken lightly as there was significant intra-class variation, as shown by the HCA dendrogram. Finally, we hope to illustrate the interesting opportunities of one-class classifier models for GC×GC-based chemometrics, as this approach is still incipient in the separation community.

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Declarations

Conflict of interest The authors declare no competing interests.

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