Chemometric Algorithms for the Direct Determination of Lipids in Synthetic Mixtures and Human Serum

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Although polyunsaturated fatty acids (PUFAs) can be detected by chromatographic methods, a low cost method to quantify PUFAs in serum samples with little to no separation or sample preparation would enable PUFAs to be monitored and detected during routine clinical serum analysis. The goal of this research project was to develop a simple, direct alternative method for the determination of the PUFAs in addition to cholesterol in human serum. The simple colorimetric assay used is rapid, rugged, inexpensive, and specific to the -C=CH-CH_2- group that accomplishes, in a single assay the simultaneous quantitation of cholesterol, ω-3 (methyl esters of linolenic, eicosapentaenoic (EPA) and docosahexaenoic (DHA)) fatty acids, and ω-6 (methyl esters of linoleic, conjugated linoleic (CLA), and arachidonic) fatty acids. Several chemometric models consisting of K-matrix using ordinary least squares (OLS) and non-negative least squares regression (NNLS), ridge regression K-matrix (RR), P-matrix regression (PM), principal component regression (PCR), and partial least squares (PLS) were introduced and applied for the direct determination of lipids in synthetic human serum models. The principal outcome was that the RR, PM, PCR, and PLS algorithms successfully out-performed the K-matrix regression approach when applied to the study of prepared mixtures (synthetic sera) in chloroform solutions. In the case of assays for intact human serum specimens, the same PLS model yielded results for ω-3 and ω-6 polyunsaturated fatty acids (PUFAs). These data compared very well for the same samples when measured using the gas chromatography-mass spectrometry (GC-MS) gold standard method. Similar results were also derived from the between-methods ω-6/ ω-3 ratios. This study has also demonstrated how chemometric algorithms might provide alternatives to separations methods for the direct determination of lipids in human serum and its synthetic models. The obvious profits from this accomplishment are the reductions in time and costs.

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Introduction

Hypercholesterolemia, obesity, coronary heart disease, diabetes, metabolic syndrome, insulin resistance, and cancer are all health conditions that are of major priorities in research laboratories and are often discussed in the news in Westernized societies. Cholesterol and polyunsaturated fatty acids have become a focus in the biomedical area in the evaluation of risk factors for the abovementioned diseases. Polyunsaturated fatty acids have been examined for positive and negative effects on the conditions listed above and many others. ω-6 fatty acids such as the linoleic, conjugated linoleic, and arachidonic acids are known to increase the cases of cardiovascular disease, hypertension, non-insulin dependent diabetes

mellitus, obesity, cancer, and myocardial infarction [1-3]. ω-3 fatty acids such as the α-linolenic, EPA, and DHA, on the other hand, are essential for normal growth and development and may play an essential role in the prevention and treatment of hypertension, coronary heart disease, arthritis, inflammatory and autoimmune disorders, diabetes, cancer, and arthritis [4-10]. The trend in the Western diet has been shifted from a low to a high ratio of ω-6/ω-3 fatty acids [11]. The alteration to this ratio and the increased rates of the abovementioned diseases have raised questions focusing on the possibility of an association between these two observations [11].

There are several descriptions in the literature for the determination of cholesterol and polyunsaturated fatty acids in human serum. For polyunsaturated fatty acids, the current methods for the determination include gas chromatography (GC), thin layer chromatography (TLC), and high performance liquid chromatography (HPLC) [12]. However, these methods are complicated, quite labor-intensive, and it is difficult to obtain meaningful concentrations when using these methods. No references were found for the direct determination of cholesterol and poly-unsaturated fatty acids by spectrophoto-metry and chemometrics.

A broad search is underway of simultaneously detecting cholesterol and polyunsaturated fatty acids in human serum in a cheaper and faster way. Based on the assay previously developed by the Purdie laboratory [13], an attempt of simultaneously determining the molar concentrations of these analytes in human serum is attempted. With appropriate experimental designs, several chemometric models consisting of K-matrix OLS, NNLS, RR, PM, PCR, and PLS are attempted and consequently compared for the determination of the mentioned analytes in synthetic mixtures (human sera). An initial attempt of using PLS in human serum was done and the ω-6/ω-3 ratios are validated with GC-MS.

Theories regarding the abovementioned chemometric models can be referred to some references in this article [13-20] and will not be discussed further.

Materials and Methods

Human Serum Samples

Serum samples for this work were provided by staff and volunteers at the Hillcrest Medical Center (HMC) in Tulsa, Oklahoma. The anonymous samples from HMC were from volunteers who were already requesting a lipid profile and had given consent. No attempt was made to solicit samples nor was any extensive medical information derived from the samples except for the cholesterol concentration which was determined by an outside clinical lab using an enzymatic test. Subjects fasted for at least 12 hours prior to the collection of the sample. A venous blood sample was collected into a Vacutainer™ red and grey capped separation tube. After inversion of the tube five times to mix the blood and the components of the collection tube, the sample was centrifuged at 3400 RPM for 15 minutes. The collection tube contained a clotting activator which takes approximately 30 minutes to activate and a floating gel that separates the red blood cells from the serum during the centrifugation step. The serum, which was the top layer in the tube, was then transferred to a 10 ml glass vial with a screw cap. The experimental assay was completed within three days of receiving the sample. Samples were stored in a refrigerator at

2-4°C and were allowed to return to room temperature prior to analyses. HMC samples were drawn from patients with normal to elevated cholesterol levels.

For serum sample analysis, a 10 µl sample of serum was added to a 13 x 100 mm borosilicate disposable test tube. 1 ml of 98 % acetyl chloride (AC) (Acros) was added to the test tube. A 40 µl aliquot of perchloric acid (PA) (70% ACS reagent grade, GFS) was carefully added down the inside of the test tube and slowly introduced to the acetyl chloride, sample solution. The reaction starts on first contact with the perchloric acid. The solution was shaken by hand for twenty seconds to allow for the release of the small amount of HCl (g) from the reaction test tube. The test tube was covered with a Teflon cap and placed into a centrifuge and spun for 3 minutes at 3400 RPM. After centrifugation, precipitated proteins were separated, and the reagent solution was transferred to a 10 mm pathlength optical glass cuvette that was fitted with a Teflon stopper for the remaining time. Absorbance spectra were measured after 15 minutes on an HP8452A Hewlett Packard spectrophotometer. A 5 second integration time and 2 nm spectral resolution were used to collect the absorbance data over the range of 350-550 nm. The blank for each reaction was pure acetyl chloride. The reagent mixture of acetyl chloride with perchloric acid did produce a slight color at 15 minutes. Due to the possibility of variability and small absorbance value, the combination of acetyl chloride and perchloric acid was not used as a blank.

Synthetic Mixtures

Methyl esters of ω-6 fatty acids (linoleic, conjugated linoleic, arachidonic), ω-3 fatty acids (α-linolenic, eicosapentaenoic, docosahexaenoic) and free cholesterol in chloroform solutions were all used to prepare synthetic mixtures to be used as training and prediction sets. The training set was done using a full factorial design (n=128), and the prediction set was done using D-optimal design (n=16) using the *SAS-JMP Software Package* [21].

All of the standards were 90 to 99 % pure based on gas chromatographic analysis and were all purchased from Sigma-Aldrich. Stock solutions for each of the analytes with maximum total concentrations of 0.02 M and 0.04 M were prepared. The stock solutions were used to prepare mixtures to limit the maximum spectral response to ranges between 0.2 and 0.9 absorbance units.

The inclusion of water was taken into account in this study. Serum normally consists of 97 % water [22]. With the sample size of serum being 10 µl, approximately 9.7 µl of water was added to the reagents in cases where synthetic mixtures are analyzed. The final experimental assay involved the addition of 10 µl of distilled water as the first step, followed by 1 ml AC, 10 µl chloroform mixture sample, and finally 40 µl PA. The final steps of the assay remained the same as serum in order to maintain constancy during the 15-minute reaction period.

Chemometric Analyses

Mean centering was not opted by the authors in this study as a common preprocessing step for the spectroscopic data due to issue that calibrations produced with mean-centered data can respond to small instrumentation drifts by generating large errors in predicted concentrations [23].

K-matrix OLS, NNLS, RR, PLS, and PCR calculations were done in MATLAB using *Chemometrics Toolbox* [24].

Determining the optimum number of factors (rank) to be used in the calibration is a key step in both PCR and PLS. To select the number of factors for PLS and PCR methods, the cross-validation, leaving out one sample at a time, was used. This process was repeated 127 times, until each sample had been left out once. The Predicted Residual Error Sum of Squares (PRESS) was used to determine the optimum number of factors in both algorithms. To calculate the PRESS we compute the errors between the expected and predicted concentrations for all of the samples, square them, and sum them together as given by the equation (1) below [20]:

$$PRESS = \sum_{i=1}^{N} (y_i - y_i')^2 \qquad (1)$$

where *y* and *y'* are the predicted and actual concentrations and *N* is the number of samples. The logarithmic plot of the PRESS values as a function of the number of factors indicates the rank to be used in the calibration.

The root mean square error (RMSE) is also calculated for each algorithm. The general equation is

$$RMSE = \sqrt{\frac{\sum_{i=1}^{N} (y_i - y_i')^2}{N}} \qquad (2)$$

The model with the minimum values for the root mean square error can indicate the appropriate model. In this paper, all PLS calculations refer to PLS2 after an initial comparison showed that PLS2 yielded lower root mean square error of prediction (RMSEP) than PLS1.

Gas-Chromatographic (GC-MS) Quantitation of Serum Samples

Validation was done by quantitating the same serum samples using GC-MS detection. Blood serum was esterified using the method given by Guy Lepage and C. Roy [25]. 1 µl of the upper benzene phase of the esterified serum was chromatographed as methyl esters on 30-m fused silica column with an internal diameter of 0.320 mm. The column was wall-coated with 0.25 mm DB-23. Analysis was performed on a Shimadzu (GCMS-QP2010) gas chromatograph. Helium was used as the carrier gas. The injection temperature was held at 250^{o}C and the column oven temperature of 50^{o}C. Splitless injection mode was used and the oven temperature program was held for 2.0 minutes at 50^{o}C and then raised 180^{o}C at 10^{o}C/min and after 5.0 minute hold, the temperature was raised to 240^{o}C at a rate of 5.0^{o}C/min and held for 13 minutes. Peaks were identified by the use of pure reference compounds. Six polyunsaturated fatty acids (PUFAs) from 18 to 22-carbons were identified.

Results and Discussion

Figure 1 shows the comparison of the RMSEP for the seven different algorithms in each lipid analyte as calculated according to equation (2). It is very clear that the NNLS algorithm when applied to K-matrix yielded lower RMSEPs than their ordinary regression approaches. It is well known that the introduction of physically induced constraints reduces the error amplification factor of so-called incorrectly posed problems (highly sensitive to measurement errors), sometimes by an order of magnitude [26-27]. Gayle and Bennet [28] showed examples demonstrating the advantages of NNLS. Jochum and Schrott [29] also showed in their study the striking advantage of NNLS and its reliability of the

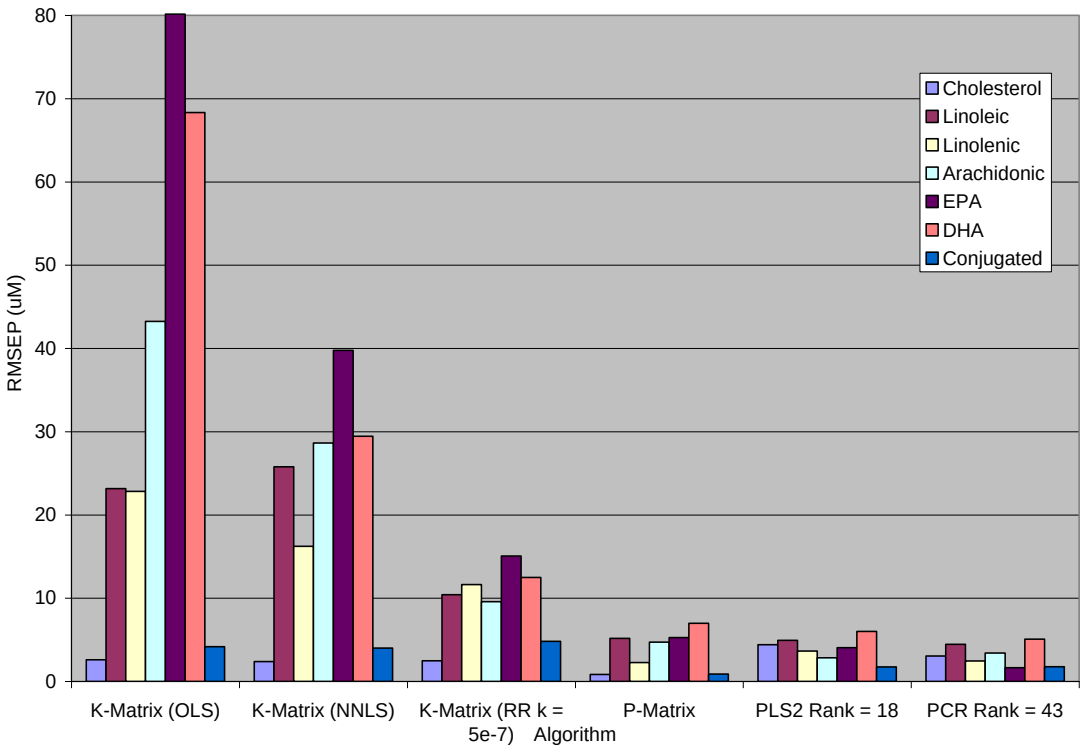

Figure 1. RMSEP comparison for each algorithm comparing the different lipid analytes.

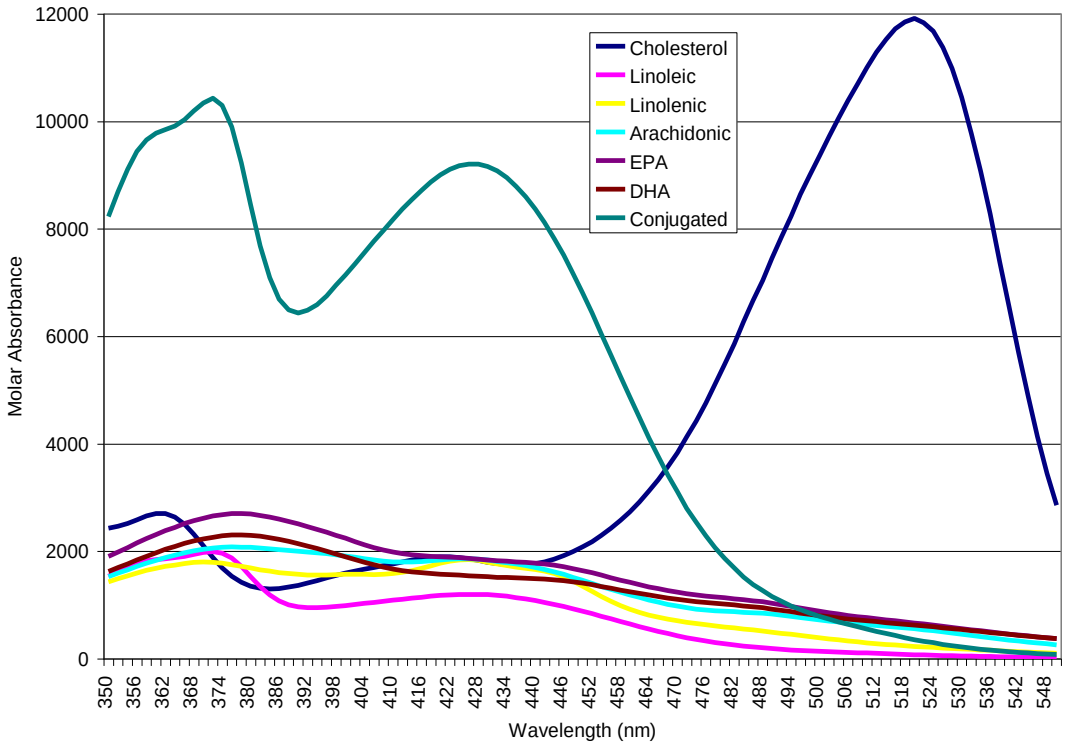

Figure 2. Molar absorptivities of the seven lipid analytes determined by the K-matrix model.

computed amounts of constituents at low concentrations.

The large RMSEPs contributed by the K-matrix approach is very evident in Figure 1. Though this approach offers the advantage of representing the genuine absorptivities with reference to the spectra of the individual constituents as shown in Figure 2, it does, however, have the disadvantage in that the calibration and analysis are connected to the inversion of a matrix [30].

Although this is not a problem from the point of view of computational time, it might become a problem if ill-conditioned (less selective) systems are applied, where the spectra of the constituents are very similar [15]. In Figure 2, it is evident that several constituents have similar spectra save for cholesterol and conjugated linoleic. The RR technique was attempted in order to improve the prediction errors in such cases. The ridge parameter obtained by using the value of k=5.00E-7, which is the value taken from the plot of the standardized ridge coefficients vs. ridge parameter (Figure 3) resulted in improved results in the RMSEPs over the ordinary K-matrix least squares solution as shown in Figure 1. In a comparative simulation study by Frank and Friedman [31], it was shown that often the RR performs as well as PCR or PLS, all of them outperforming multiple linear regression (MLR) with forward variable selection.

An alternative to the K-matrix approach is to calibrate the concentrations directly on the spectra. This is known as the P-matrix approach (or inverse model). A disadvantage of this calibration method is that the calibration coefficients (elements of the P-matrix) have no physical meaning, since they reflect the spectra of the individual components. Figure 4 shows the P-matrix regression coefficients obtained from the seven lipid analytes.

It is evident in Figure 1 that the P-matrix technique also yielded comparable results with that of PCR and PLS. P-matrix approach offers a slight advantage over the classical K-matrix approach because a second matrix inversion is avoided [15].

One of the assumptions made in MLR is that the independent variables are truly independent. To the degree that this assumption is invalid, the resulting model parameters will be more affected by noise, eventually leading to loss of full rank [32]. Attempts to eliminate this collinearity problem have led to such developments as PCR and RR.

Among all algorithms attempted, P-matrix, PCR, and PLS performed quite equally well, exhibiting low RMSEP values. The number of factors in PCR in Figure 1 might be high enough but this number of factors was determined to be the optimum number after cross validation calculations.

PLS is a powerful multivariate statistical tool that has been successfully applied to the quantitative analysis of ultraviolet [33, 34], near-infrared [35-38], chromatographic [38-40], and electrochemical data [41]. PLS offers the signal-to-noise advantage gained by making use of all the measurements. Furthermore, by using only the significant number of latent variables in the procedure, a noise filtering effect is obtained which results in its improved predictive stability [42]. In a study conducted by Cassel ,et al [43] wherein PLS was tested in the presence of three inadequacies, (i) skew instead of symmetric distributions for manifest variables; (ii) multi-collinearity within blocks of manifest and between latent variables; and

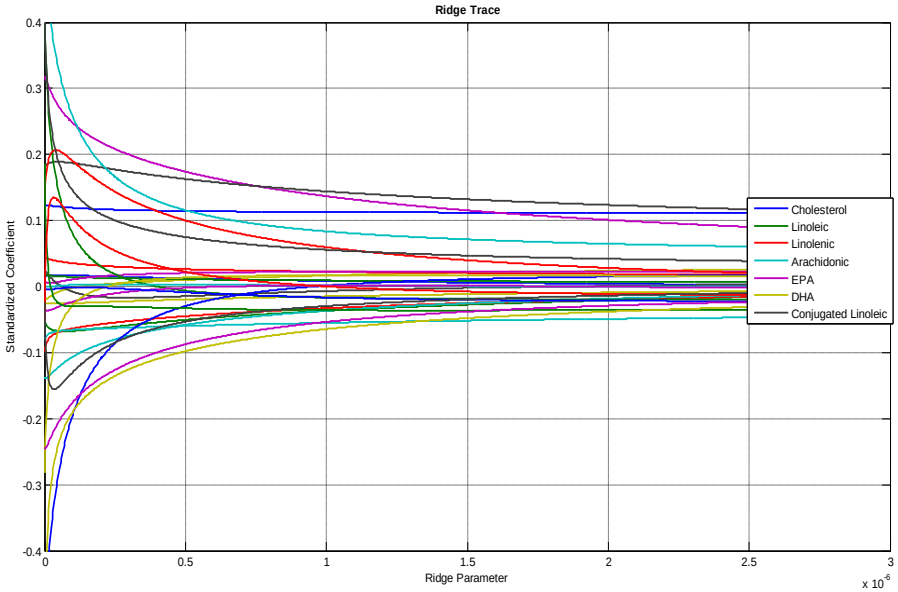

Figure 3. Plot of standardized coefficient vs. ridge parameter for the RR approach. The point at which the ridge parameter, k = 5.00E-7, represents the optimum parameter value leading to lowest RMSEPs.

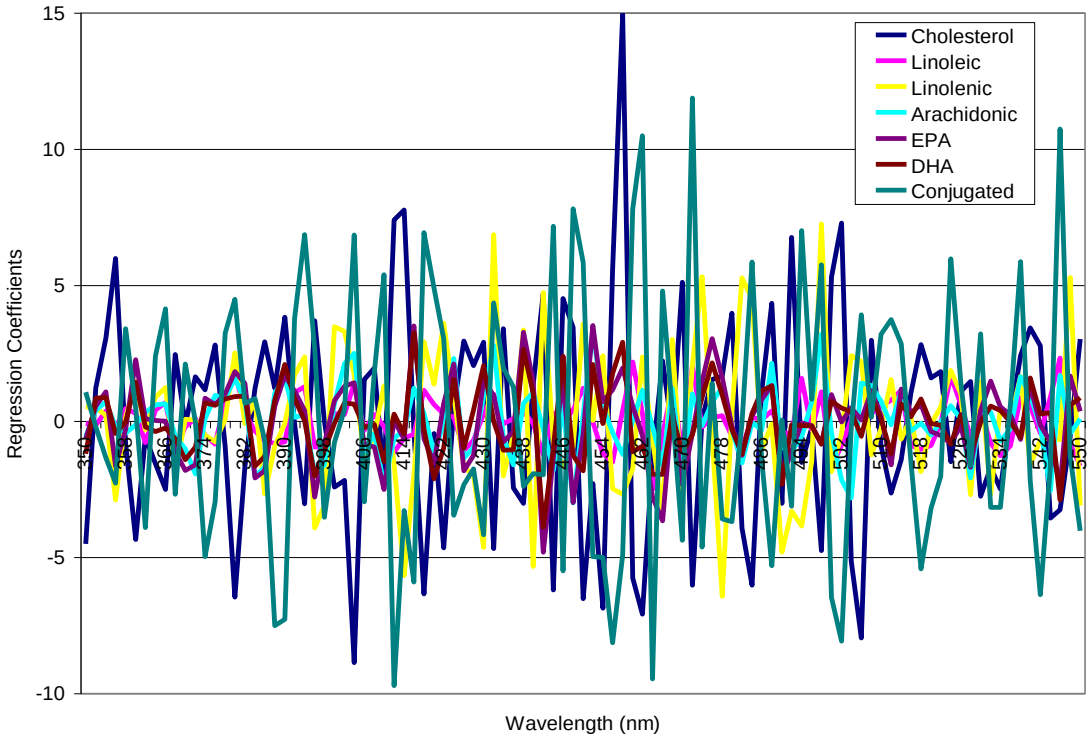

Figure 4. P-matrix regression coefficients obtained by using the P-matrix approach.

(iii) misspecification of the structural model (omission of regressors), the algorithm showed quite a robustness in these three inadequacies.

An alternative figure showing how the different constituents behave with respect to the different algorithms is shown by their RMSEPs

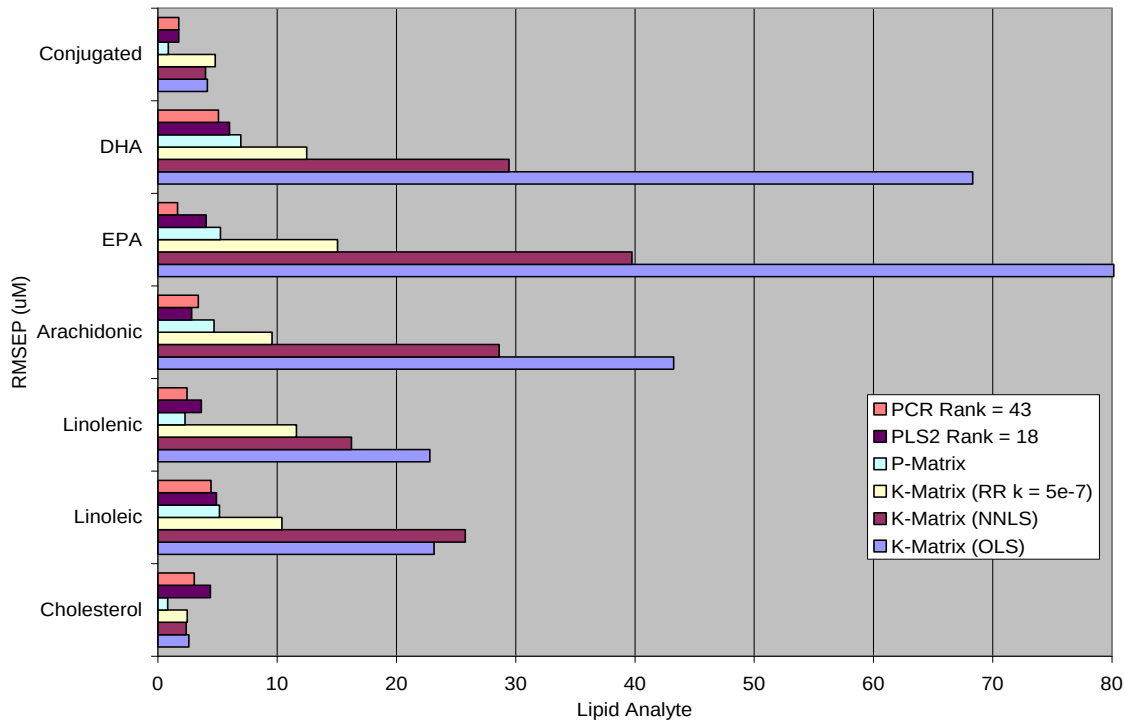

Figure 5. RMSEPs for the different algorithms as clustered in each lipid analyte.

Table 1. Comparing ω-6 total conc % and ω-3 total conc % between PLS and GC-MS of five serum samples.

Category	ω-6 total conc %	ω-6 total conc %	ω-3 total conc %	ω-3 total conc %	ω-6 /ω-3 total conc %	
Patient's code	PLS	GC-MS	PLS	GC-MS	PLS	GC-MS
P1	47.86	47.17	52.14	52.83	0.92	0.89
P2	50.12	54.05	49.88	45.95	1.00	1.18
P3	49.59	46.59	50.41	53.41	0.98	0.87
P4	48.83	47.93	51.17	52.07	0.95	0.92
P5	49.05	48.22	50.95	51.78	0.96	0.93

Table 2. Comparing cholesterol PLS and enzymatic test of five serum samples.

Sample	Cholesterol, PLS pred. (mg/dL)	Cholesterol, Enzymatic (mg/dL)	Percent Error
P1	203.61	187	-8.88
P2	207.27	188	-10.25
P3	187.98	189	0.54
P4	193.31	189	-2.28
P5	174.05	163	-6.78

in Figure 5. Clearly from the figure, cholesterol and conjugated linoleic yielded the lowest RMSEPs. As mentioned earlier, these analytes have distinctive characteristic spectra that could be clearly distinguished from the other lipid analytes (Figure 2). EPA, DHA, linolenic, and arachidonic exhibit similar spectra and, thus, yielded high RMSEPs.

The PLS2 calibration coefficients were tested on some serum samples. The same serum samples were validated using GC-MS. Table 1 shows the comparison for the total ω-6 and ω-3 PUFA concentrations expressed as percentages and their ratios. It can be noted that the ω-6and ω-3 total % concentration and the ratio of ω-6 to ω-3 were substantially identical between the two methods. Table 2 shows the comparison between the PLS and the standard enzymatic test for cholesterol.

Summary and Conclusions

A number of independent chemometric algorithms were tested that included MLR-NNLS, RR, PCR, and PLS. The principal outcome was that the RR, P-matrix, PCR, and PLS algorithms performed quite equally well enough than the K-matrix approach when applied to the study of prepared mixtures (synthetic sera) in chloroform solutions. The PLS model was tested for intact human serum specimens, and yielded results for ω-3 and ω-6 PUFA data that are comparable when using the GC-MS gold standard method. Similar results were also derived for the between-methods ω-6/ω-3 ratios. In this study, therefore, the dominance of PLS over the other chemometric models has been shown. This paper has also shown how Friedel-Crafts acylating assay coupled with chemometric algorithms might provide alternatives to separations methods for the direct determination of lipids in human serum and its synthetic models. The advantages of this simple technology are the reduction in time and costs.

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