Partial Least Squares (PLS1) Algorithm for Quantitating Cholesterol and Polyunsaturated Fatty Acids in Human Serum

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We have previously exploited various chemometric algorithms for the direct determination of cholesterol and polyunsaturated fatty acid (PUFA) molar concentrations in synthetic mixtures and human serum. The simple colorimetric assay used is rapid, rugged, inexpensive, and specific to the -CH=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). Previously, ridge regression (RR), P-matrix regression (PM), principal component regression (PCR), and partial least squares (PLS2) successfully out-performed the K-matrix regression (KM) approach when applied to the study of prepared mixtures (synthetic sera) in chloroform solutions. In this paper, partial least squares in the form of PLS1 is investigated and applied to quantify molar concentrations of cholesterol and PUFAs in actual human serum samples. Results show that PLS1 yielded lesser root mean square errors of prediction in the calibration model, and molar concentrations comparing quite equally well with the gas chromatography-mass spectrometry (GC-MS) procedures.

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Introduction

In the modern era, biomedical research plays a very critical role in human health. Within the biomedical research area, scientists are searching for new biomarkers that would serve to identify the causes of obesity, coronary heart disease, diabetes, hypercholesterolemia, and cancer among others. Cholesterol and PUFAs are among the biomarkers associated with the previously mentioned diseases. PUFAs in the diet have long been considered essential to the growth and proper nutrition of humans and animals. On the contrary, they have also exhibited negative effects [1]. PUFAs exist in two major kinds, the ω-6 and the ω-3 forms. The ω-6 fatty acid esters such as the linoleic, conjugated linoleic, and arachidonic acids are known to enhance formation of cholesterol gallstones, a stimulus to carcinogenesis, increased vitamin E requirements, promotion of obesity, increased uptake of plant sterols, and increased cholesterol absorption [1-3]. The ω-3 esters of the fatty acids such as α-linolenic, EPA, and DHA, on the other hand, have effects on diverse physiological processes impacting normal health and chronic disease, such as the regulation of plasma lipid levels, cardiovascular and immune function, insulin action, and neuronal development and visual function [4-14].

The ω-6/ω-3 ratio is an important indicator of human health. There is evidence that a 4:1 ratio

is required for maximum benefit for cardiovascular disease and less than 2:1 to have any effect on cancer [15].

Genetically speaking, human beings today live in a nutritional environment wherein major changes in our diet have taken place, particularly in the type and the amount of essential fatty acids and in the antioxidant content of foods [16, 17-20]. Comparing the hunter-gatherer with the western diet and lifestyle, the ω-6 to ω-3 ratio has shifted considerably from low to high [18]. Excessive amounts of ω-6 PUFA and a very high ω-6/ω-3 ratio, as is found in today's Western diets, promote the pathogenesis of many diseases, including cardiovascular disease, cancer, and inflammatory and autoimmune diseases, whereas increased levels of ω-3 PUFA (a lower ω-6/ω-3 ratio), exert suppressive effects [21].

Currently, several methods exist for the determination of cholesterol and PUFAs levels in human serum. Gas chromatography (GC), thin layer chromatography (TLC), and high performance liquid chromatography (HPLC) are the methods commonly used for PUFAs level determination in human serum [22]. However, these methods are complicated, quite laborious, and suffer from the difficulty of obtaining meaningful concentrations.

The Purdie Assay was established to enable the simultaneous quantification of cholesterol and PUFAs in synthetic mixtures and human serum without the need for analytical separations [23]. The assay originated with the Liebermann-Burchard reaction that was once the current gold standard for cholesterol, and was later based upon a reaction attributed to Chugaev and Gastev. The assay reagent had the extra selectivity of acylation of the α- over the ß-position at the C-17 carbon that enabled the differentiation of anabolic steroids. In that and a following study, it was also determined that if multiple unsaturated lipids are present, the resulting compounded spectrum is the simple addition of the weighted spectra for each of the components, assuming no extraneous interferences are present [24].

Using this assay, various training set models for the simultaneous quantitation of cholesterol and PUFAs in synthetic mixtures and human serum were reported [17]. After exploitation of several chemometric models including KM, PM, RR, PCR, and PLS2, our studies showed that PLS2 yielded results for ω-3 and ω-6 PUFA data that are comparable when using the GC-MS method. Similar results were also derived for the between-methods ω-6/ω-3 ratios [25].

In this paper, PLS in the form of PLS1 is reported for obtaining the actual molar concentrations of cholesterol, and fatty acids of linoleic, linolenic, arachidonic, EPA, DHA, and conjugated linoleic in human serum. The training set data were mean centered prior to performing the said algorithm. The results are then compared with the GC-MS method. The RMSEP of the PLS1 algorithm is also compared with the other algorithms. In addition, neural network (NN) was also attempted in the training sets and has been included in this paper.

The theories behind the various chemometric algorithms will not be further discussed but can be referred to some bibliographic references in this paper [26-32].

Materials and Methods

Human Serum Samples

The staff and volunteers at the Hillcrest Medical Center (HMC) in Tulsa, Oklahoma provided us with the human serum samples. Such anonymously named samples were from individuals who had requested a lipid profile and had given consent. No attempt was made to solicit samples nor was any extensive medical information derived from the samples. Prior to sample collection, the subjects fasted for at least 12 hours, and using a Vacutainer™ red and grey capped separation tube, venous blood samples were collected from these individuals. 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 AC, 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 then 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. This wavelength range was chosen for the reason that the lipid analytes exhibit spectral variations in this range. For the analysis, the visible spectrum obtained for a typical plasma sample turned out to be the linear sum of the weighted contributions from all seven analytes that – given the heterogeneity of blood samples – leads to a broad diversity in the spectral patterns [23]. The blank for each reaction was pure AC. The reagent mixture of AC with PA did produce a slight color at 15 minutes. The combination of AC and PA was not used as a blank, due to the possibility of variability and small absorbance value out of such solution mixture.

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* [33]. 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 [34]. 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 in serum in order to maintain constancy during the 15-minute reaction period.

Chemometric Analyses

Deconvolution of the mixture absorbance spectra to obtain the lipid analyte concentrations was done by applying chemometric algorithms. As compared to the previous paper, mean centering is applied to both PLS and PCR algorithms in this paper. Mean centering was also attempted in KM, RR, and PM but generated root mean square errors of predictions (RMSEPs) which are much larger than the non-mean centered training data sets. Accordingly, the training data sets were not mean-centered in KM, RR, and PM regression models. Chemometric analyses were performed in MATLAB using *Chemometric Toolbox* [35]. Neural network was performed using the *JMP Software Package* [36].

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 [35]:

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

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 RMSE indicated the appropriate model.

Gas Chromatography-Mass Spectrometry (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 [37]. 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°C and the column oven temperature of 50°C. Splitless injection mode was used and the oven temperature program was held for 2.0 minutes at 50°C and then raised 180°C at 10°C/min and after 5.0 minute hold, the temperature was raised to 240°C at a rate of 5.0°C/min and held for 13 minutes. Peaks were identified by the use of pure reference compounds. Six PUFAs from 18 to 22-carbons were identified.

Results and Discussion

Neural network (NN) was first attempted in this study. Using three hidden nodes, four number of tours, and with a 0.01 overfit penalty, the RMSEP in the training model is still considerably higher than any other algorithms. Though NNs can implicitly detect complex non-linear relationships between independent and dependent variables, they suffer from disadvantages of being prone to "overfitting," and are "black box" and have limited ability to identify possible causal relationships [38].

As with the previous results wherein PLS2 outperformed all other algorithms in the training model [25], partial least squares in the form of PLS1 yielded lesser RMSEP than PLS2 in the same training model in this paper after mean centering of the training data set (Figure 1). In PLS1, the highest RMSEP is obtained for DHA. The possibility of similarity in the molar absorbance spectra for EPA and DHA would be the reason why the RMSEP is higher for DHA (Figure 2). Nevertheless, this results show that despite similarities in the molar absorbance of the lipid components, the RMSEP of all components using PLS1 is still low as compared to other algorithms.

PLS2 differs from PLS1 in the way used to perform the signal decomposition and the regression analysis. PLS2 calculates the number of factors on all the components simultaneously and one weighed number of factors is optimized. PLS1 performs the optimization of the number of factors for only one component at a time. The application of PLS in spectroscopic data can be referred to some bibliographic references [39-43].

Choosing the optimum number of factors in PLS1 is the key to obtain a good calibration model. The trick is to keep only those factors that contain analytical information. The discarded factors should contain only noise. If too many factors are kept, there is danger of overfitting the data and adding noise to the calibration. If there are not enough factors, a proper calibration model cannot be generated [35].

From Figures 3 and 4, it is readily apparent that prediction errors are minimized when calibrations are developed using the indicated number of factors as stated in the analyte's respective figure captions. The obtained PLS2 and PLS1 calibration models were applied to five serum samples obtained from HMC. Save for conjugated linoleic, all lipid components yielded positive molar concentrations in PLS1. As compared to the previous paper wherein PLS2 used 18 factors [25], the factors were reduced to an optimum number of 6 in PLS2 in this paper. The possibility of including a wide range of cholesterol and PUFAs concentration ranges calibration matrix is still collected, and when done, this assay will serve as a direct, time and cost saving method for simultaneously quantitating cholesterol and PUFAs in human serum.

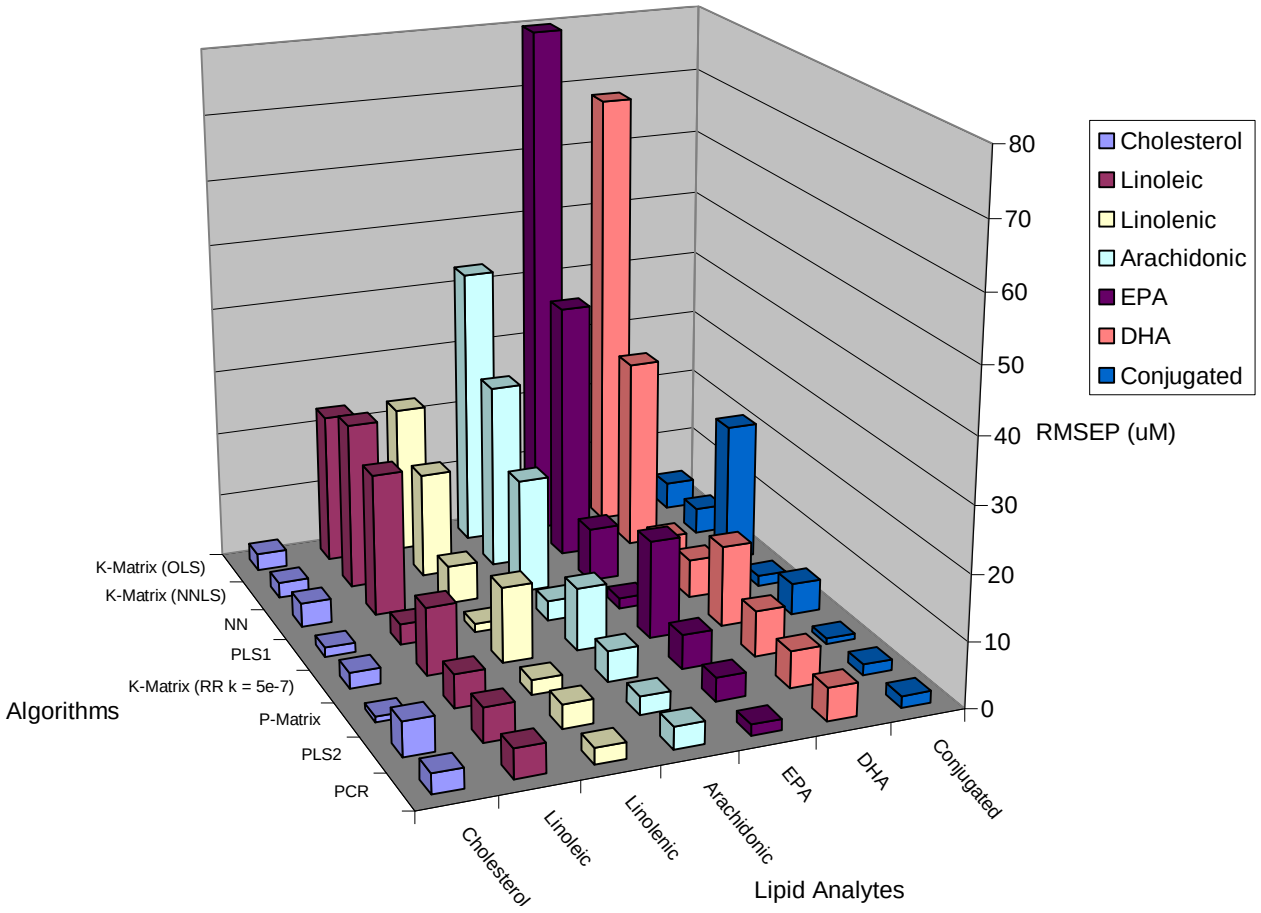

Figure 1. 3-dimensional (3D) diagram of the RMSEP of the lipid analytes in each algorithm. PLS1 yielded the least RMSEP for all analytes. Other algorithms are identical as in the previous paper and are shown for comparison purposes only (25).

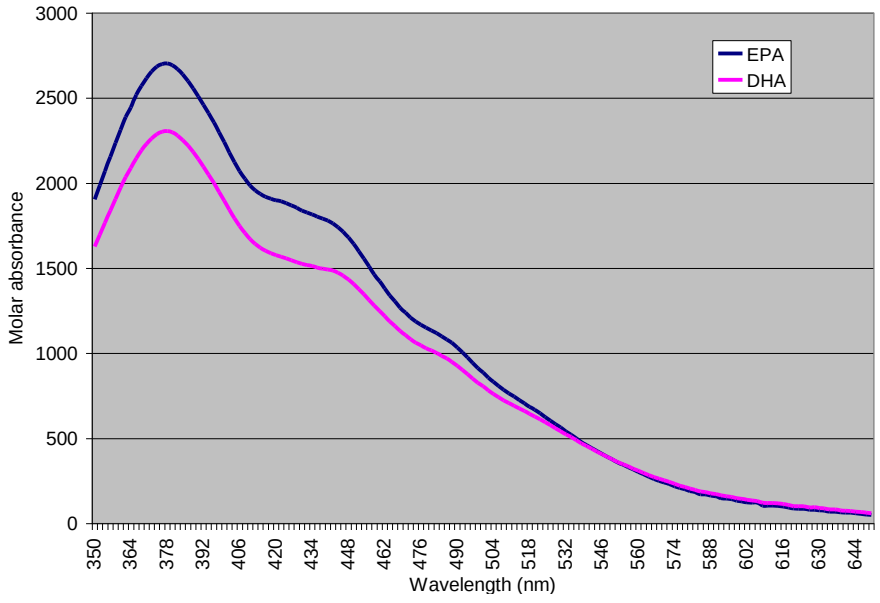

Figure 2. Molar absorptivities of EPA and DHA determined by the K-matrix model as in the previous paper (25). These are shown for comparison purposes only.

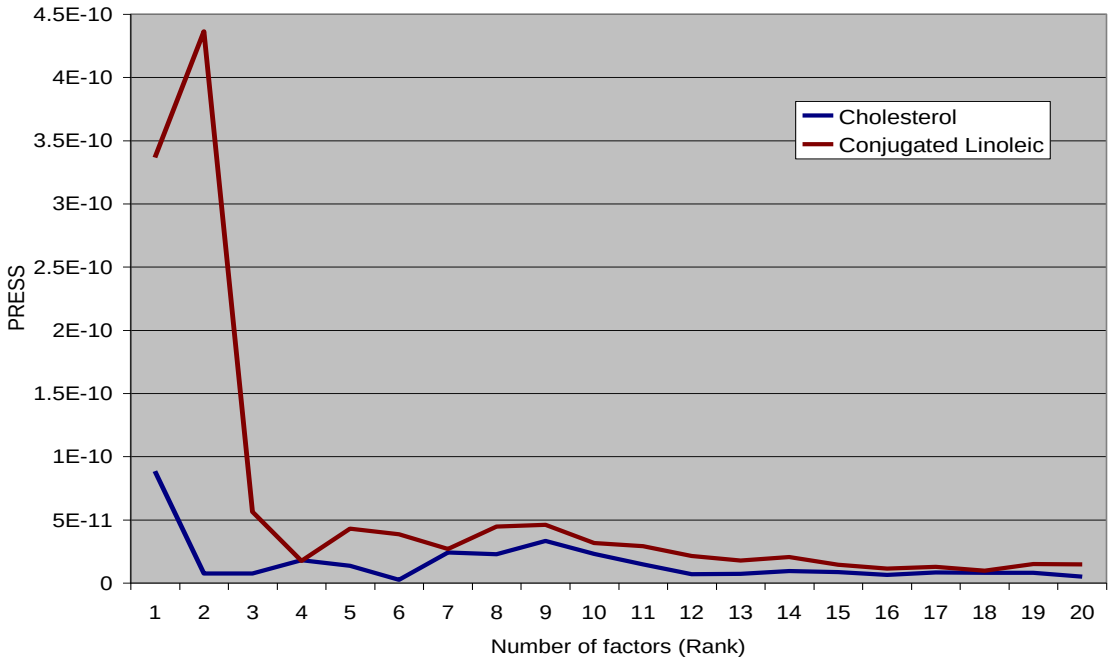

Figure 3. Plot of PRESS vs ranks for cholesterol and conjugated linoleic. 6 and 4 factors were chosen for cholesterol and conjugated linoleic, respectively.

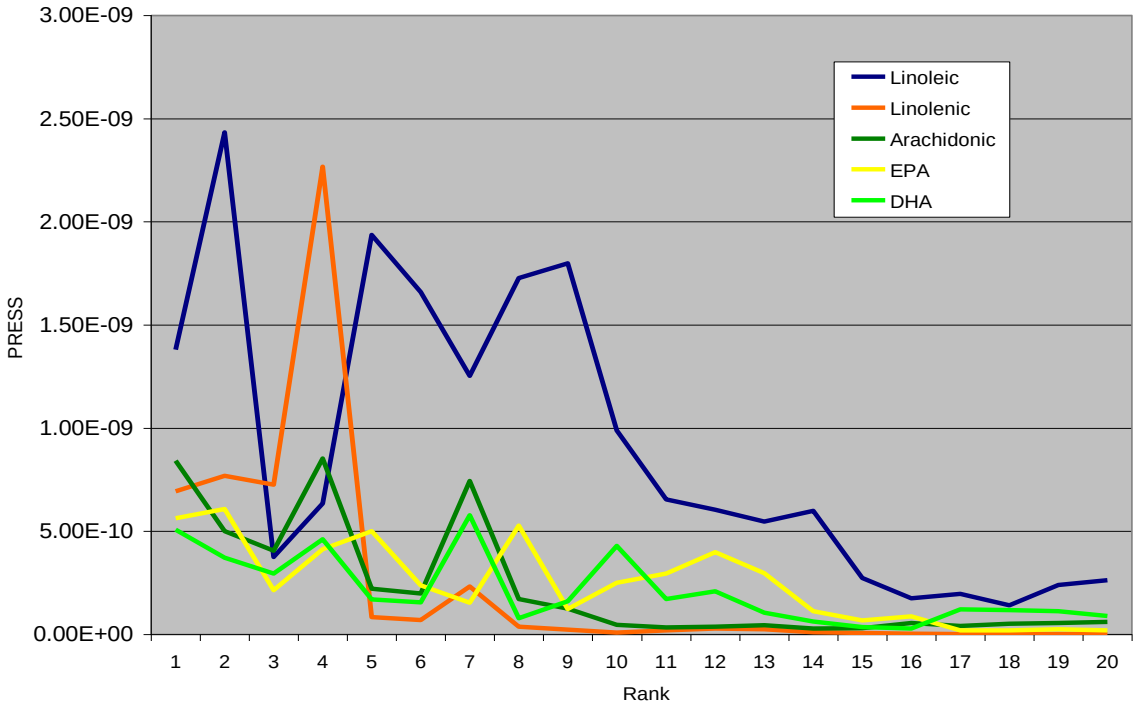

Figure 4. Plot of PRESS vs ranks for linoleic, linolenic, arachidonic, EPA, and DHA with ranks 3, 8, 17, 7, and 3, respectively.

In Table 1, molar concentrations of cholesterol, linoleic, linolenic, arachidonic, EPA, and DHA compared quite equally well with the GC-MS method. In Table 2, molar concentrations of conjugated linoleic compared quite equally well with the first two samples but not with the

Table 1. PLS1 molar concentrations of cholesterol, linoleic, linolenic, arachidonic, EPA, and DHA in human serum samples compared to GC-MS.

	PLS1	GC-MS	% Difference	PLS1	GC-MS	% Difference
	Cholesterol			Linoleic		
P1	4.21E-03	4.09E-03	2.89	2.08E-03	1.98E-03	4.89
P2	3.25E-03	3.01E-03	8.13	3.27E-03	2.87E-03	14.0
P3	2.25E-03	2.39E-03	6.05	2.87E-05	3.19E-05	9.88
P4	3.13E-03	3.39E-03	7.55	2.62E-03	2.44E-03	7.18
P5	3.11E-03	3.19E-03	2.41	2.06E-03	3.01E-03	31.4
	Linolenic			Arachidonic		
P1	3.13E-05	2.67E-05	17.2	1.46E-03	1.50E-03	2.42
P2	3.83E-05	3.48E-05	10.0	2.86E-04	2.52E-04	13.7
P3	5.79E-04	5.31E-04	9.05	2.40E-03	2.31E-03	3.84
P4	5.76E-05	6.10E-05	5.58	2.86E-04	2.52E-04	13.7
P5	3.75E-05	6.16E-05	39.1	9.70E-05	1.38E-04	29.7
	EPA			DHA		
P1	2.83E-03	3.52E-03	19.5	2.28E-03	2.73E-03	16.5
P2	4.05E-03	4.30E-03	5.86	9.77E-04	1.05E-03	6.91
P3	2.39E-03	2.97E-03	19.6	1.80E-03	1.83E-03	1.81
P4	2.10E-03	1.85E-03	13.3	1.74E-03	2.77E-03	37.1
P5	2.17E-03	2.20E-03	1.39	1.80E-03	1.60E-03	12.3

Table 2. PLS1 molar concentrations of conjugated linoleic in human serum samples compared to GC-MS.

	PLS1	GC-MS	% Difference
P1	1.22E-04	1.21E-04	0.67
P2	5.10E-04	5.17E-04	1.39
P3	-8.26E-05	5.70E-04	-
P4	-4.12E-04	1.03E-04	-
P5	-5.63E-04	7.05E-05	-

remaining three. CLA in normal physiological human serum exists in low concentrations (10-70 μM) as compared to other fatty acids, linoleic (2270-3850 μM), α-linolenic (50-130 μM), arachidonic (520-1490 μM), EPA (14-100 μM), and DHA (30-250 μM) [44, 45]. This would be the most probable reason of the negative molar concentrations for the PLS1 in CLA.

Although successful, especially, in comparing relative percentage change in fatty acids for clinical studies, GC's disadvantages include the derivatization steps which can alter the structure of the fatty acid or create side-products that can overlap with the analytes needed [46]. Short chain fatty acid methyl esters can be eluted quickly and missed [47]. Also, the procedures are quite labor intensive; and it is difficult to obtain meaningful concentrations when using only a limited number of standards. These limitations strengthen the case for the development of this

simple and direct method assay that does not require separation and reacts directly with the PUFAs.

Summary and Conclusions

As with the previous research results wherein PLS2 outperformed KM, PM, RR, and PCR in prepared mixtures in chloroform solutions (synthetic sera), PLS1 yielded the least RMSEP for all the lipid components as compared to all other algorithms in this study. This study has also attempted to determine the molar concentrations of cholesterol and PUFAs in human serum by the PLS1 algorithm. PLS1 yielded molar concentrations quite comparable with the GC-MS method in the actual human serum samples. The consistencies in the validation are evidence that the assay can be used as an alternative to the GC-MS procedures. While the GC-MS procedures gives only percentage values of the PUFAs, and obtaining a calibration curve in terms of peak areas and heights is a very tedious task, this new spectroscopic technology offers the advantages of being direct, simple, rapid, and cost efficient. The assay has a potential market for a wide array of clinical settings wherein GC-MS is impossible.

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