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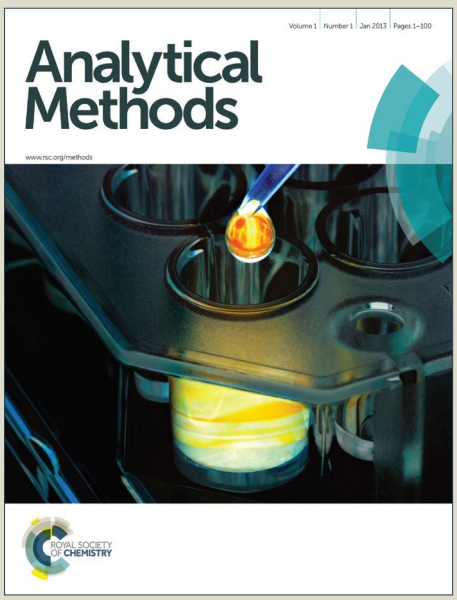

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Cardiovascular biodiagnosis by infrared spectroscopy through choline determination

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In this work a green analytical method has been proposed for diagnosis of heart disease. In this method infrared spectroscopy has been employed for quantitative determination of choline as an important correlated biochemical in blood samples. Attenuated total reflection Fourier-transform infrared (ATR-FTIR) spectroscopy has been used for analysis of blood serum samples while the spectrometric data was processed by partial least squares (PLS). In the experimental step 82 blood serum samples were studied at 4000–600 cm−1 spectral region. Preprocessing methods such as standard normal variate (SNV) and multiplicative scatter correction (MSC) were utilized with no evidence on the analytical output, while orthogonal signal correction (OSC) could affect the accuracy of the method severely. The RMSEP for the OSC-PLS model was 0.39%, thus it could be considered as an appropriate data processing strategy for ATR-FTIR spectrometric determination choline in serum blood samples. It is rapid, reliable, non-destructive and free of sample preparation or chemical reagent consumption and it is called as a green diagnostic approach.

Introduction

The main text of the article should appear here with headings as appropriate. Cardiovascular diseases are the leading cause of deaths worldwide. According to available statistics, about 40 percent of the deaths are caused by cardiovascular diseases in the developing countries e.g. Iran. One of the main criteria for health monitoring is to evaluate the performance function of human heart. There are several experimental methods for diagnosis of cardiovascular defects while some of these methods are invasive and expensive [1]. Thus there is a serious demand for development of fast and accurate methods which provide efficient approaches for diagnosis of cardiovascular related health problems. Early diagnosis of heart disease and cardiovascular defects may help in prevention of heart attack. Thus there is a serious demand for development of reliable approaches which would help in early and robust diagnosis of cardiovascular related health problems.

Efforts in development of diagnostic methods of heart disease have been powerfully aimed to introduce the reliable techniques with more figures of merit. Over the years, there have been several reports dealing with biochemical variations as a sign of heart defects. Increment in serum glutamate oxaloacetate transaminase of patients with acute myocardial infarction (AMI) [2,3], quantitative amount of serum lactate dehydrogenase (LDH) and serum creatine kinase(CK) [4,5], cardiac troponins [6], cardiac natriuretic peptides [7] have become as some of the available biomarker related symptoms for diagnosis of cardiac diseases.

Choline (CHO) is an essential nutrient that is usually grouped within the vitamin B complex. Choline and its metabolite betaine are methyl donors, along with folate, and are metabolically linked to transmethylation pathways including synthesis of the cardiovascular disease (CVD) risk factor homocysteine. A high plasma homocysteine concentration is associated with increased risk of CVD (Figure 1) [8].

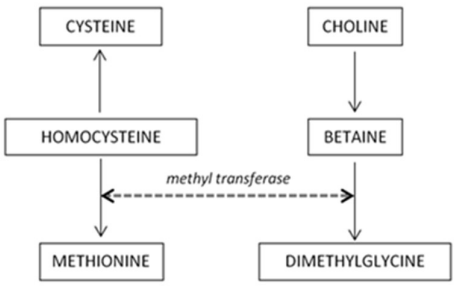

Figure 1- Schematic representation of homocysteine metabolism

Quantitative range of choline in plasma is between 7.0 and 12.3 µmol L-1 in healthy cases. Plasma choline levels are elevated in patients with acute coronary syndrome (ACS). Levels above 25 µmol L -1 have been shown to be strong predictors of cardiac events in ACS [9].

Several analytical methods have been used for quantitative determination of CHO including radioenzymatic analysis, GC-MS, LC-MS, Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) [10], capillary electrophoresis with electrochemical detection [11], rapid HPLC [12], HPLC with fluorescence detection [13] and normal-phase chromatography-tandem mass spectrometry [14].

Infrared (IR) spectroscopy has also been nominated as a powerful tool and green analytical technique in biology for studying the structure of different bio-related structures and their conformation. Analytical output of such studies can be translated in practical medical diagnosis methods [15,16]. The increasing role of mid-IR spectroscopy in biomedical research has been widely reported [17,18], for different media such as

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blood cells [19], proteins [20], cancer research [21,22] by different routes such as evaluation of information on metabolic biochemical via their IR finger print spectral features. These spectral data would be associated with different illness patterns, their stages and progression. Blood [23], blood serum [24-25], plasma and urine [26, 27] have been investigated as ideal candidates for biomedical diagnostics.

Diagnosis of disease patterns via IR spectral analysis of body fluids consists of some data processing steps. Chemometric efforts appeared to be very useful in solving many analytical problems. Multivariate chemometric data processing techniques are the main approach, applied while a research is performed to combine the statistical skills with chemical ones and obtain reliable results [28]. IR spectra are often pre-processed in order to remove systematic errors e.g. noise and base-line variation and multiplicative scatter effects.

Pre-processing is an important part of data analysis which would help in better development of robust models. There are several pre-processing approaches, employed for data treatment. Some of the most common ones are multiplicative scatter correction (MSC) which removes the scattering from spectral data, standard normal variate (SNV) which performs the scaling and centering based on standard deviation of the dataset and orthogonal signal correction (OSC) which removes unrelated or orthogonal systematic variation from the spectral data. Among these techniques, OSC has been reported to provide several benefits in thee removal and investigation of non-correlated variation contained within spectral data in spectrometric investigations [29,30].

In the present study, the ATR-FTIR spectra of serum samples obtained from normal people and those patients suffering from heart disease were evaluated to determine choline quantitatively. Role of some pre-processing techniques was investigated in development of more reliable quantitative model.

Experimental

Materials and methods

Blood samples for quantitative determination of choline were collected in 9 mL tubes containing 1 mg/mL lithium-heparin and potassium fluoride (BD, India). Whole blood samples were immediately frozen after collection at −20 °C. In order to prepare the samples for determination of choline samples, they were hemolyzed by freezing and thawing and finally centrifuged over a pre-rinsed filter (molecular mass cut-off: 10000 Da, Millipore, Germany) being de-proteinized. Plasma choline was determined from centrifuged plasma using standard sample for calibration model (Sigma, Germany). A total number of 82 samples were obtained to be investigated. The standard method for determination of choline in the prepared serum samples was based on LC-MS (Agilent Technologies, USA) with electrospray ionization in positive mode, using an ODS reversed-phase column (analytical grade, 250×3.0 mm, particle size 5µm). The mobile phase consisted of monobasic sodium phosphate (10 mmol/L), dibasic sodium phosphate (10 mmol/L), n-octylsulfate (50 mg/L) and acetonitrile (5 %v/v). The LC-MS method, validated by previously reported standard protocol [31] demonstrates the limit of quantification 0.05 µmol/L, linearity 0.5–1000.0 µmol/L, mean analytical recovery 100% and standard error of 1.4%. Attenuated total reflectance infrared (ATR-FTIR) spectroscopy analyses were carried out at room temperature by a Tensor-27 Bruker FT-IR spectrometer equipped with a Ge–KBr beam splitter, a DTGS detector and Beer–Norton apodization with a horizontal, fixed path ATR device (ZnSe, 45°), single reflection. About 0.5 ml of serum sample was placed in ATR cell. The spectral resolution was 8 cm−1 and 64 scans were accumulated over the range from 600 to 4000 cm−1 for each spectrum. Avoiding the significant role of water as the main component of samples, it was set as the spectral analysis background. The spectrum of each sample was recorded 3 times and the average was used to be processed. Wavelength penetration and baseline corrections were utilized. Data manipulation was by OPUS and the obtained spectra were treated by polynomial baseline correction. Chemometric data processing was performed by using MATLAB Ver. 8.0 (The MathWorks Inc., MA, USA).

Chemometric data processing

In order to extract the most informative features of the spectral data set, a pre-processing strategy was considered in this work. In the first step, data set was mean centered to remove the constant error. Mean centering would subtract the data set mean from each data entity. As the output quantitative analysis of choline in serum samples by the chemometric techniques is influenced by the magnitude of the spectral expressions, mean centering is helpful due to removal of the dependence on magnitude, providing a mean expression of zero for the data set. This would enable the model to consider the relative changes, instead of the absolute magnitudes. In the next step, standard normal variate transformation was performed to reduce the baseline shift and collinearity. Multiplicative scatter correction (MSC) was the other employed transformation to compensate for additive and multiplicative effects in spectral data. Orthogonal signal correction (OSC) was also utilized to remove the information unrelated to the target variables based on constrained principal component analysis. Detection of outliers based on principal component analysis (PCA) and data splitting by Kennard-Stone algorithm were performed and the prepared spectral data set was introduced to the partial least squares (PLS) calibration model to investigate the role of initial pre-processing on the reliability of final quantitative model.

Results and discussion

Informative assignment of IR spectra

Assigning the IR signals in a typical serum spectrum, there are several informative spectral features in the investigated spectral region correlated to different functional groups. Some of the most important assigned bands in this spectral region (600–4000 cm−1) are detailed in figure 2 and table 1 [32-34]. Choline is (2-Hydroxyethyl)trimethylammonium and as a quaternary ammonium salt, its cation is a phospholipid. Its main correlated spectral characteristics have been shown in figure 2 and table 1 in grey colour.

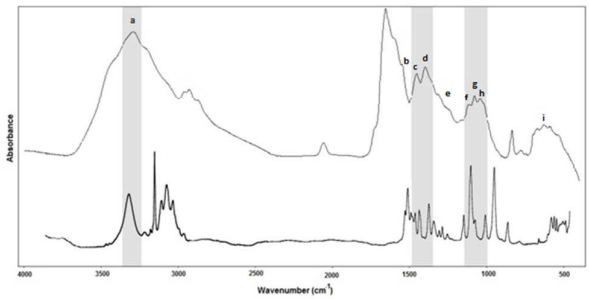

Figure 2- Typical ATR-FTIR spectrum of serum sample in which the main spectral features have been assigned (top) and spectrum of choline (bottom)

Table 1- Assignment of common ATR-FTIR spectral features in their related functional groups in serum samples (those correlated with choline structure have been shown by grey shade)

	vibration	Spectral region (cm^{-1})	bio chemical structure
a	N-H stretching	3300	amide A
b	C–N stretch., N–H def.	1550	amide II, protein
c	CH_2 sym. def.	1470	lipid
d	coupled C–H/N–H def.	1290	amide III, protein
e	O–P=O anti-sym. stretch.	1235	DNA, RNA, phospholipids
f	C–O stretch, C–O–H bend	1150	carbohydrates, mucin
g	O–P=O sym. stretch.	1120	DNA, RNA, phospholipids
h	–C–O–P stretch	1070	phophodiester group, DNA, RNA
i	S-S bending	650	disulfide bond

Detection of outliers

Outlier detection is an important task in data analysis. Outlier detection is to detect objects which do not resemble the bulk of the dataset. In the field of fraud detection, network intrusion detection, etc., outlier detection is a very critical task as outliers usually indicate a threat to the integrity of the system. And because of the insufficient knowledge and inaccurate representative of the outlying objects in a given system, outlier detection is also interpreted as one-class classification problem, where a one-class classifier tries to model a representation of the normal data so as to identify outliers which do not fit the model. The most common procedure for this aim is to assign atypical objects before modelling. In this regard, the original set of sample spectra, the vector of responses and score plots on the first principal components (PC) must be considered. The leverage of each sample is recommended to be examined in the X space (spectral data space), to detect possible outliers. Leverage is a measure of a sample's spatial distance to the main body of the samples in the data space. Special attention must be paid to those data points with high leverage because of their strong influence on parameter estimation which may alter the model severely in case of being an outlier. Considering the first 3 PCs up on implementation of leverage method, 13 samples were flagged as outliers and thus the models were built by the remaining 69 samples. The assigned 3 PCs (Figure 3) would cover 99.97% of total variance of data set. Performing PCA to dataset, the first 3 PCs explained 99.08, 0.16 and 0.02% of variance respectively.

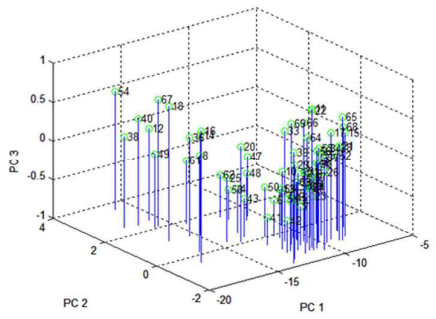

Figure 3- Score plot of PCA on spectral data set for serum samples

Quantitative determination of choline by Partial least squares (PLS) regression

PLS is a linear modelling technique, successfully adopted in many quantitative assays. Variables are factors, calculated during the processing procedure and describe the maximum amount of information for concentration matrix. Decomposition of latent variables is the basis for development of regression models while the number of latent variables would influence the predictive capability of the model. The developed model is used to predict the concentration of unknown samples. Leave one out cross validation is the route to evaluate the built model. PLSR has been extensively used in modelling of infrared spectra. Near infrared spectroscopy (NIRs) in combination with partial least squares is an effective, expeditious and non-destructive technique to analyse various parameters of interest to the pharmaceutical industry [35], compounds in injection [36], human serum samples[37], food industry [38] and many other titles.

Using Kennard-Stone algorithm a set of 48 samples was selected for calibration by PLS and the remaining 21 samples formed the prediction set. MSC, SNV and OSC were conducted on the data set separately and the role of pre-processing on predictive capabilities of PLS model was investigated. In order to validate the model and realize the optimum number of factors (ONF), the cross validation was repeated, leaving out one of the samples in each round until each calibration sample left out once. The predicted concentration of choline in each sample was compared with its known concentration and the root mean square of cross validation (RMSECV) was calculated. The number of factor which yields minimum RMSECV is a reasonable choice for optimizing the model. Various PLS calibration models have been calculated for the samples of the training set using different "number of factors". RMSEP values estimate the absolute errors of prediction and the model's reliability for the analyte and by this strategy, ONF equal to 4 was considered for the whole dataset.

$$RMSEP=(\sqrt{(\sum(Ci-\hat{C}i))2/ N} \quad (1)$$

where N is the number of samples, Ci is the real concentration of the component and $\hat{C}i$ is the estimated concentration.

Role of multiplicative scatter correction (MSC) on PLS

MSC is a mathematical treatment to correct the scattering effect in the spectral data. The light scattering or change in path length for each sample is estimated relative to that of an ideal sample. In principle this estimation should be done on a part of the spectrum which does not contain chemical information, i.e. influenced only by the light scattering. However the areas in the spectrum that hold no chemical information often contain the spectral background where the SNR (signal-to-noise ratio) may be poor. This correction would make the same scatter level for all spectra as the ideal. The theoretical expression of MSC is to perform the best if an offset correction is carried out first, as:

$$xi = a + b\, x_{ij} + ex \quad (2)$$

where xi is the IR spectrum of the sample, and $\bar{x}j$ is the desired spectrum of the ideal sample (the mean spectrum of the calibration set). For each sample, a and b are estimated by ordinary least-squares regression of spectrum xi vs. spectrum $\bar{x}j$ over the available wavelengths. Each value xij of the corrected spectrum xi (MSC) is calculated as:

$$Xij\ (MSC) = (Xij-a)/b\ ;\ j=1,2,\ldots,p \quad (3)$$

The mean spectra must be stored in order to transform in the same way future spectra. Performing the MSC on the data set, R2 and root mean squared error of prediction (RMSEP) for the MSC-PLS model were 0.7631 and 62.89% respectively. Thus more exclusive pre-processing must be considered.

Role of standard normal variate (SNV) on PLS output

SNV is a pre-treatment used quite often in infrared spectrometry on individual spectra instead of each wavenumber to centre and scale data by their own standard deviation. During

the SNV transformation, average and standard deviation of all the data points for a spectrum is calculated and the average value is subtracted from the magnitude (e.g. absorbance intensity in the IR spectra) for every data point and the result is divided by the standard deviation. "R" has a function to centre and scale every vector which we can use to get the SNV spectrum. Performing the SNV transformation prior to PLS regression R^2 and root mean squared error of prediction (RMSEP) for the MSC-PLS model were 0.9069 and 47.27% respectively. SNV seems more effective than MSC in case of linear regression, however the error in the prediction set is not reasonable at all.

Orthogonal signal correction (OSC)

OSC is a pre-treatment to remove systematic variation from the spectral data matrix that is unrelated, or orthogonal, to the choline concentration matrix based on constrained principal component analysis. This may enhance the predictive power and lower the complexity of the resulting PLS model, and obtain a great simplification in terms of model interpretation. It is important to be assured that the analyte is retained. In this work, OSC algorithm was utilized in an attempt to reduce quantitative model complexity by removing orthogonal compounds from the signal. It was used as a pre-processing step prior to latent variable regression modelling in PLS to remove the structured noise in X (spectral data matrix). OSC model is expressed as:

$X = t_{osc}\, pT_{osc} + X'$ (4)

where $t_{osc} = X_{wosc}$ and Y T $t_{osc} = 0$. Here, tosc, posc, and wosc represent the single OSC component. X' is the OSC-filtered matrix subsequently used in the PLS latent variable regression model. Several OSC components may be identified and removed from spectral data matrix. Identified OSC components possess 2 sets of loading vectors, being similar to the PLS components, but the score vector tosc is orthogonal to Y. Different OSC filters are usually compared based on number of OSC components removed. It is difficult to make an assured comparison because one OSC component can be derived from different multicomponent prediction models [39-40]. The model developed on OSC pre-processed data was by single latent variable, compared to three latent variables for the models fitted to the SNV and MSC pre-processed data.

In case of the calibration set for PLS regression, OSC components were used for filtering. Statistical evaluation of the prediction errors of the validation set in quantitative determination of choline reveals that the OSC treated data provide substantially higher reliability of prediction values than the original data, MSC-PLS and SNV-PLS. Statistical parameters for all three models are detailed in table 2.

Table 2- Comparison between different pre-processing strategies in quantitative determination of choline (outliers removed prediction set)

	SSE	R2	adjusted R2	RMSE (%)
SNV	4.25 ×10-4	0.907	0.902	47.27
MSC	7.52 ×10-4	0.763	0.751	62.89
OSC	0.012	0.996	0.995	0.25

Sum of square error measures the total deviation of the predicted choline concentration values from the fit to the predicted values. It is also called the summed square of residuals and is calculated as:

SSE = Sum(i=1 … n){wi (yi - fi)2} (5)

where yi is the observed value and fi is the predicted value from the fit. wi is the weighting applied to each data point, usually wi = 1. Adjusted R2 is a statistical parameter indicating the fit quality when the predicted data and standard concentration data are compared. Root mean square error (RMSE) is the fit standard error and the standard error of the regression. It is an estimate of the standard deviation of the random component in the data:

$RMSE = s = (MSE)^{½}$ (6)

where MSE is the mean square error or the residual mean square

MSE=SSE/v (7)

As mentioned previously, 13 samples had been flagged as the atypical cases, called outlier and thus, they had been removed from the data set. However, in case of medical evaluation tests, it is necessary to obtain a determination procedure which has the most compatibility with all types of samples, even the outliers. In the other words, for a medical examination, it is not favourable and convenient to leave some of the obtained data from medically collected samples with no response. In order to evaluate the capabilities of the proposed model in evaluation of all serum samples, the remaining 13 outliers where also added to the prediction test set. The comparative demonstration of statistical parameters for the quantitative determination methods (Table 3), verifies the powerful prediction ability of the proposed technique. Presence of outliers labelled samples in the data set would slightly increase the errors, which is negligible. Figure 4 shows the actual vs. predicted values for all 3 models.

Table 3- Comparison between different pre-processing strategies in quantitative determination of choline in whole data set (outliers added to prediction set)

	SSE	R^2	adjusted R^2	RMSE (%)
SNV	4.28×10^{-4}	0.769	0.759	43.17
MSC	7.82×10^{-4}	0.630	0.614	45.77
OSC	0.018	0.994	0.990	0.39

Dealing with medical diagnostic approaches, sensitivity and specificity are two important statistical parameters, determined to measure the performance of the diagnostic classification model. Trying to differentiate between healthy and patient cases, sensitivity measures the proportion of actual positives which are correctly identified while specificity measures the proportion of negatives which are correctly identified. These two parameters determine how useful the test is to detect a disease or characteristic in the given population. In the other words, sensitivity relates to the test's ability to identify positive results:

Sensitivity = number of true positives / (number of true positives + number of false negatives)

And specificity relates to the test's ability to identify negative results:

Specificity = number of true negatives / (number of true negatives + number of false positives)

Predicting the choline concentration in 82 samples (11 patients and 71 healthy samples) for diagnostic aims, all the patient cases were predicted correctly while 3 healthy samples were misclassified. Thus sensitivity and specificity were 100% and 95.77% respectively.

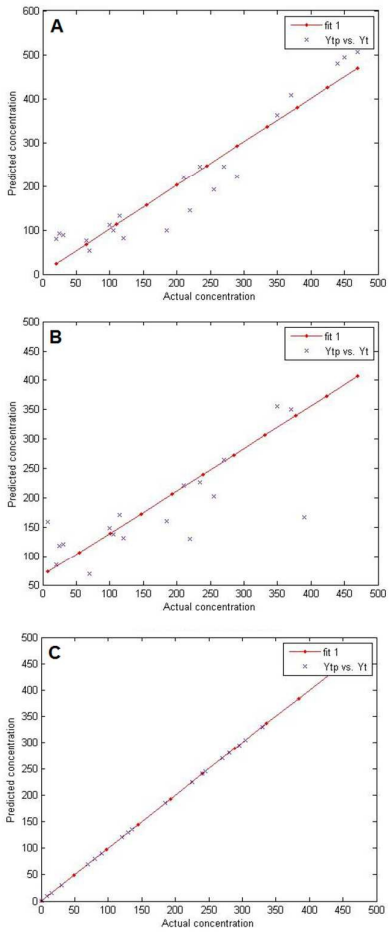

Figure 4- Actual vs. predicted values of choline for (A) MSC, (B) SNV and (C) OSC methods

Green Approaches

FTIR Spectroscopy is known as a rapid reliable analytical instrument which could provide robust qualitative and quantitative outputs for different analytical goals. There are 12 principles for green analytical chemistry and in order to call a method as a part "Green Analytical Chemistry, it is necessary to explain its compatibility with these principles. The ATR-FTIR based approach proposed in this work is:

- a direct analytical procedure which could be employed, avoiding any sample treatment.
- possible to be performed on minimal sample size while the robust chemometrics model allows the analysis to be conducted by minimal number of samples
- one step analysis which saves time and energy
- reagent free analysis
- capable of automization by different routes e.g. FIA
- free of analytical waste
- possible to be employed for evaluation of different biochemical analyses with in the serum sample and called multi-parameter methods
- free of any toxic reagent or intermediate
- safe and hazardless for the analyst

Analysis of the error

The boundary phase of choline concentration in serum samples for diagnosis of cardiovascular defects is 25 µmol.lit-1 and this has been considered as the borderline in the quantitative analysis. One of the major concerns in biodiagnosis via quantitative determination is the rate of prediction error and its distribution along the concentration evolution. As observed in figure 5 the proposed OSC-PLS model for quantitative determination of choline is independent of the analyte concentration and the prediction error is distributed normally across the sample concentration.

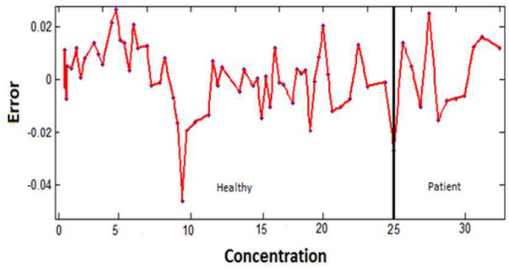

Figure 5- Distribution of prediction error in quantitative determination of choline

Conclusions

In this paper combination IR spectrometry and chemometrics approaches as green analytical techniques could help in development of a rapid, simple and non-invasive diagnosis method for cardiovascular defects. Three processing methods were compared in PLS based calibration model for quantitative determination of choline in spectral data: SNV, MSC and OSC. Considering the RMSE and coefficient of determination in the calibration model, in case of OSC-PLS the reliability is more than two are techniques. The results demonstrate that ATR-FTIR is feasible as a useful green analytical tool for rapid and simple preliminary diagnosis for heart disease. The results of this study clearly show the OSC method would remove the information unrelated to the target variables based on constrained principal component analysis.

1 Sato, Y.; Fujiwara, H.; Takatsu,Y. *Journal of Cardiology* , 2012,**59**, 1.
2 Karmen, A.; Wrobleski, F.; LaDue, J.S. *The journal of clinical investigation*, 1955, **34(1)**, 126.
3 LaDue,J.S.; Wrobleski, F.; Karmen, A. Science, 1954, **120**, 497.
4 Amador, E.; Dorfman, L.E.; Wacker, W.E. *Clinical Chemistry*, 1963, **9**, 391.
5 RJ. Dunn, AL. Siegel, *Archives of Internal Medicine*, 1965, **115** , 443.
6 Katus, H.A.; Remppis, A.; Looser S.; Hallermeier, K.; Scheffold, T.; Kübler, W. *Journal of Molecular and cellular Cardiology*, 1989, **21**, 1349.
7 Cowie, M.R.; Mendez, G.F. *Progress in Cardiovascular Diseases*, 2002, 44, 293.
8 Dalmeijer, G.W.; Olthof, M.R.; Verhoef, P.; Bots, M.L.; van der Schouw. Y.T. *European Journal of Clinical Nutrition*, 2008, **62** , 386.
9 Danne, O.; Lueders, C.; Storm , C.; Frei, U.; Möckel, M. *Clinica Chimica Acta*, 2007 , **383**, 103.

10 Persike, M.; Zimmermann, M.; Klein, J. *Analytical Chemistry*, 2010 , **82** , 922.
11 Wise, D.D.; Barkhimer, T.V.; Brault, P.A.; Kirchhoff, J.R.; Messer, W.S.; Hudson, R.A. *Journal of Chromatography B*, 2002, **775**, 49.
12 Webb, L.E.; Pavlina, T.M.; Hjelle, J.T. *Clinical Biochemistry*, 1993, **26**, 173.
13 McEntyre, C.J.; Slow, S.; Lever, M. *Analytica Chimica Acta*, 2009 , **644**, 90.
14 Holm, P.I.; Ueland, P.M.; Kvalheim, G.; Lien, E.A. *Clinical Chemistry* , 2003, **49**, 286.
15 Mantsch, H.H.; Chapman, D. *Infrared Spectroscopy of Biomolecules*. Wiley , NewYork, 1996.
16 K. Brandenburg, U. Seydel, *Chemistry and Physics of Lipids*, 1998 , 96 , 23.
17 Gremlich, H.U.; Yan, B. *Infrared and Raman Spectroscopy of Biological Materials*, Marcel Dekker, Basel, 2001.
18 Moss, D. *Biomedical applications of synchrotron infrared microspectroscopy. A practical approach. Cambridge*, UK: RSC Publishing; 2011.
19 Mostaco Guidolin, L.B.; Bachmann, L. *Applied Spectroscopy Reviews*, **2011**, 46, 388.
20 Glassford, S.E.; Byrne, B.; Kazarian, S.G. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics*, **2013**, 1834,2849.
21 Rehman, S.; Movasaghi, Z.; Darr, J.A., Rehman, I.U. Applied Spectroscopy Reviews, 2010, 45(5), 355.
22 Kelly, J.G.; Nakamura, T.; Kinoshita, S.; Fullwood, N.J.; Martin, F.L. Analyst, 2010, 135, 3120.
23 Heise, H.M.; Marbach, R.; Janatsch, G.; Kruse-Jarres, J.D. Analytical Chemistry , 1989, 61, 2009.
24 Lasch, P.; Schmitt, J.; Beekes, M.; Udelhoven, T.; Eiden, M.; Fabian, H.; Petrich, W.; Naumann, D Analytical Chemistry, 2003 , 75 ,6673.
25 Staib, A.; Dolenko, B.; Fink, D.J.; Früh, J.; Nikulin, A.E.; Otto, M.; Pessin-Minsley, M.S.; Quarder, O.; Somorjai, R.L.; Thienel, U.; Werner, G.; Petrich, W. Clinica Chimica Acta , 2001 , 308 ,79.
26 Heise, H.M.; Voigt, G.; Lampen, P.; Küpper,L.; Rudloff, S.; Werner, G. Applied Spectroscopy, 2001 , 55, 434.
27 Shaw, R.A.; Low-Ying, S.; Leroux, M.; Mantsch, H.H. Clinical Chemistry, 2000 , 46, 1493.
28 Niazi, A.; Azizi, A. Turkish journal of chemstry , 2008 , 32 , 217.
29 Artusson, T.; Hagman, A.; Bjork, S.; Trygg, J.; Wold, S.; Jacobsson, S.P. Applied Spectroscopy, 2000, 54 (8), 1222.
30 Eriksson, L.; Trygg, J.; Johansson, E.; Bro, R.; Wold, S. Analytica Chimica Acta. 2000, 420, 181.
31 Danne, O; Möckel, M; Lueders, C; American Journal of Cardiology, 2003,91, 1060.
32 Sankari, G.; Krishnamoorthy, E.; Jayakumaran, S. Biology and Medicine, 2010, 2 , 42.
33 Sankari, G.; Aishwarya, T.S.; Gunasekaran, S.; Surapaneni K.M. Journal of Clinical and Diagnostic Research, 2011, 5, 1001.
34 Sheng, D.; Wu, Y.; Wang, X.; Huang, D.; Chen, X.; Liu, X. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy, 2013, 116 , 365.
35 Luo, W.; Wu, J.; Wang, X.; Lin. X.;Li, H. Analytical Methods, 2013 , 5 , 1337.
36 Li, W.; Cheng, Z.; Wang, Y.; Qu, H. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy, 2013, 101, 1.
37 Perez-Guaita, D.; Kuligowski, J.; Quintás, G.; Garrigues, S.; de la Guardia, M. Talanta ,2013 , 107, 368.
38 Feng, Y. Z.; Sun, D.W. Talanta, 2013 , 105, 244.
39 Wold, S.; Antti, H.; Lindgren, F.; Öhman, J. Chemometrics and Intelligent Laboratory Systtem,1998, 44, 175.
40 Svensson, O.; Kourti, T.; Macgregor, J.F. Journal of Chemometrics, 2002, 16, 176.

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