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ScienceDirect
Food Chemistry 103 (2007) 1062–1068
Food Chemistry
www.elsevier.com/locate/foodchem

Analytical, Nutritional and Clinical Methods

Simultaneous spectrophotometric determination of caffeine and theobromine in Iranian tea by artificial neural networks and its comparison with PLS

Ali Reza Khanchi [a,*], Mohamad Khayatzadeh Mahani [a,b], Majid Hajihosseini [a], Mohamad Ghanadi Maragheh [a], Marzieh Chaloosi [b], Farhad Bani [a]

[a] *Jabber Ibn Hayan Research Laboratories, Atomic Energy Organization of Iran, P.O. Box 11365-8486, Tehran, Iran*
[b] *Faculty of Chemistry, Tarbiat Moallem University, 49 Mofateh Avenue, Tehran, Iran*

Received 6 March 2006; received in revised form 12 July 2006; accepted 13 July 2006

Abstract

In order to determine the amount of caffeine and theobromine, spectrophotometry was used as a simple, rapid and economical method. Because of severe overlapping between these components, artificial neural network was used. The 230–300 nm spectral window with 1 nm interval was used for data acquisition. An artificial neural network (5-5-3) with linear transfer function between input-hidden and hidden-output layers was trained and applied for prediction of concentration of these methylxanthines in four Iranian tea samples. The model was compared with PLS modeling method. HPLC technique was used as a standard method.

Keywords: Tea; Caffeine; Theobromine; Artificial neural networks; PLS

1. Introduction

The methylxanthines [caffeine (CF), theobromine (TB) and theophylline (TP)] are well-studied compounds. These alkaloids act as stimulants of the central nervous, muscle and circular systems of the human body (James, 1991). Caffeine is a widely used – and perhaps abused – psychoactive drug. It has been shown to affect every body system that is controlled by the central nervous system (Rall, 1993). Caffeine taken into the body from many different foods such as coffee and tea. The average cup of coffee contains approximately 100 mg of caffeine (Holland, Godfredsen, Page, & Corner, 1998). It is believed that drinking tea is advantageous to human health, because it contains a lot of polyphenols, which are known to have high antioxidant activity, but tea also contains caffeine, which stimulates the central nervous system and causes certain diseases (Horie, Nesumi, Ujihara, & Kohata, 2002). Determination of caffeine in tea samples is important from an economic point of view and also in the process of decaffeination of natural tea leaves. Decaffeination is useful for human health and it also produces caffeine, a valuable byproduct (Saldana, Mohamed, Baer, & Mazzafera, 1999). Horie et al. (2002) tried to bread tea cultivars that contain no or very little caffeine in the leaves. Most methods for determination of methylxanthines are chromatographic in nature (Chen, Mou, Hou, & Ni, 1998; Ikegaya, Takayanagi, & Anan, 1990; Nakakuki, Horie, Yamauchi, & Kohata, 1999). However, spectrophotometric determination in ultra-violet region is superior, because it is inexpensive and follows a simple procedure; in addition it is possible to obtain high accuracy and reproducibility rapidly from a small number of samples. Because of UV-spectral overlap between caffeine, theobromine and theophylline, many different separation procedures have been developed for determination of these components (Englis & Miles, 1954; Li, Berger, &

* Corresponding author. Fax: +98 21 88333377.
E-mail address: khanchi@gmail.com (A.R. Khanchi).

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doi:10.1016/j.foodchem.2006.07.035

Hartland, 1990; Moores & Campbell, 1948; Shingler & Carlton, 1959). However, these separation processes are difficult and even with these procedures it is impossible to quantifying methylxanthines in the same sample simultaneously. In recent years, attention has been directed toward chemometrics methods for analysis of multi-component systems because of fast data collection using rapid scanning spectrophotometers. Aksu, Bozdogan, and Kunt (1998) determined caffeine and theobromine in cocoa by partial least squares multivariate spectrophotometric calibration. Martinez, Alba, Campos, and Rodriguez (2003) used PLS modeling for simultaneous determination of caffeine, theobromine and theophylline in tea samples. Chemometrics based on artificial neural network (ANN) have found increasing applications for multi-component determinations, especially in the nonlinear calibration systems.

There has been no report on using artificial neural networks for simultaneous determination of methylxanthines in tea samples. In this work, a spectrophotometric method has been developed for simultaneous analysis of ternary mixtures of methylxanthines (caffeine, theobromine and theophylline) in synthetic samples and four types of Iranian tea samples with applying principle component analysis (PCA) and ANN. In this paper, partial least squares regression (PLS) was also compared with principle component artificial neural network (PC-ANN).

2. Materials and methods

2.1. Apparatus

A Cary 3E UV-Vis double beam spectrophotometer (Varian Company, Melbourne, Australia) was used to record absorption spectra. A magnetic stirrer MR 3001 K (Heidolph Company, Schwabach, Germany) was used for mixing and pH was adjusted using a pH-meter CG 841 (Schott Company, Mains, Germany). A high performance liquid chromatograph (HPLC) with binary pump, dual wavelength absorbance detector and Nova Pak C-18 reverse phase 5 μm 150 mm × 4.6 mm column (all from Waters Company, Milford, MA, USA) was utilized for the analysis of the samples over an 8 min period. An aqueous mobile phase containing 80% buffer (0.015 M potassium phosphate pH 4.9) and 20% methanol with 1.0 ml min^{-1} flow rate was used and the detector was set at 274 nm.

2.2. Reagents

All chemicals were used with analytical-grade from Merck (Darmstadt, Germany) or Sigma-Aldrich (Saint Louis, MO, USA), and doubly distilled water was used throughout the work. Stock solutions of caffeine, theobromine and theophylline were prepared by dissolving appropriate amounts of each compound in distilled water. A Britton–Robinson buffer solution pH 12 was prepared by mixing 0.04 M of each boric, orthophosphoric and acetic acids with 0.2 M of sodium hydroxide (Dean, 1995). Standard solutions of these methylxanthines or their mixtures were then diluted to the required concentrations in 15 ml volumetric flask with 2 ml of buffer solution and appropriate distilled water.

2.3. Software and data processing

PLS-NIPALS algorithm, PCA and ANN programs were written in MATLAB (v. 6.5 for windows). All programs were run on a PC (Pentium IV 3.00 GHz) with Microsoft Windows XP operating system.

2.4. Sample preparation for spectrophotometric analysis

Four types of tea leaves were collected from two different regions (Lahijan and Langrood in the northern of Iran). Green tea leaves were dried at 95 °C in 100 mmHg vacuum. Green and black samples were crushed in a porcelain mortar to make uniform powder. One gram of each powder was weighed and was poured into 100 ml of water for 30 min at 100 °C. The extract was then filtered and the filtrate was diluted in 100 ml volumetric flask with distilled water. Twenty five milliliters of this solution were mixed with 0.5 ml of lead acetate (10%w/v), and the mixture was stirred for 2 min at room temperature. The resultant solution was filtered and 0.1 ml of sodium carbonate (10%w/v) was added to the filtrate to remove excess lead acetate. The mixture was filtered and diluted in a 100 ml volumetric flask with distilled water (Martinez et al., 2003).

2.5. Partial least squares

The partial least squares regression method (PLS) is a quantitative spectral decomposition technique that is closely related to principle component regression (PCR). However in PLS, the decomposition is performed in a slightly different fashion. Instead of first decomposing the spectral matrix into a set of eigenvectors and scores, and use regression of them against the concentration as a separate step, PLS actually uses the concentration information during the decomposition process. Non-linear iterative partial least squares (NIPALS) algorithm for PLS was used in the present work. In brief, PLS breaks down both the measured and concentration matrices into a product of two smaller matrices. In this process, PLS actively uses the variables included in the concentration matrix as the data matrix is being broken down. It involves a data compression step where the measured data are compressed to a smaller number of intensities, called "scores", in a new coordinate system. The new coordinate axes are called latent variables (LVs) or factors and are linear combinations of the original variables. A LV represents a systematic variation found in the data set. The regression coefficients from each original variable to each LV are called

"loadings". PLS assumes the concentration to be a linear function of scores. PLS-1 performs an individual component regression of the concentration to the new latent variable and PLS-2 does such regression for all components simultaneously. PLS-1 was used for this work because, it provides better result for real samples (Vandeginste et al., 1998).

The number of factors to be used within the PLS-1 algorithm is an important parameter to achieve better performance in prediction. This allows modeling of the system with the optimum amount of information and avoidance of over-fitting or under-fitting. The leave-one-out validation and segmented cross-validation procedures consisting of systematically removing one or a group of the training samples in turn, and using only the remaining ones for the construction of the latent factors and regression were applied. The predicted concentrations were then compared with the actual ones for calibration of tea samples and the predicted error sum of squares (PRESS) was calculated. The PRESS was computed in the same manner and each time a new factor was added to the PLS model.

$$\mathrm{PRESS} = \sum_{i=1}^{I} \sum_{j=1}^{J} (C_{i,j} - \hat{C}_{i,j})^2$$

where $\hat{C}$ is the analyte estimated and C is the added concentration for the mixture. i and j indices are for sample and component, respectively.

2.6. Artificial neural network

ANN is an abstract simulation of a real nervous system that contains a collection of neuron units communicating with each other via axon connections. Such a model bears a great similarity to axons and dendrites in a nervous system. ANNs "learn" from examples, and exhibit some capability for generalization beyond the training data. Many different types of ANNs have been developed. They all consist of small units, neurons that are interconnected. Each neuron contains input, weights associated with each input, transfer function and output. Weights are considered as the distributed knowledge content of the network, therefore the weights (+ or −) are essential. One of the various types of ANNs is Back Propagation (BP) Networks. The structure of the BP algorithm comprised of three layers, contain input, output and hidden layer. BP networks sometimes are called multilayer feed forward (MLF) networks because of There is no signal propagation within a layer or to a previous layer. ANNs can be applied for linear and non-linear calibration and modeling. The ANN model is defined by the number of neurons (nodes) in the input, hidden and output layers. The number of predictor or independent variables and predicted or dependent variables determines the number of nodes in input and output layers respectively (Zupan & Gasteiger, 1993; Sekulic et al., 1993).

The ratio of the number of samples to the number of adjustable parameters in the ANN should be kept as large as possible. One way of over determining the problem is to compress the input data, especially when they consist of absorbance recorded at several hundred wavelengths. In addition to reducing the size of input data, compression allows one to eliminate the irrelevant information such as noise or redundancies present in the data matrix. Successful data compression can result in increased training speed, a reduction of memory storage, better generalization ability of the model and enhanced robustness with respect to noise. The most popular method for data compression in chemometrics is principle component analysis (PCA). Most ANN Applications in quantitative analysis with spectral data use PC scores obtained from PCA, as input variables.

Principle component-artificial neural network (PC-ANN) has good speed and low calibration error, because of reducing the input data for ANN (Gemperline, Long, & Gregoriou, 1991). In this type of networks, principle component analysis was first applied to data and then the scores on the selected principle components were subjected to ANN as input.

3. Results and discussion

3.1. Design of mixtures

Two trimerous set of synthetic mixtures containing all three methylxanthines present in random ratio were prepared; one set with 25 samples for training and one set with 5 samples for prediction. Due to severe matrix effect even in pretreated tea samples, 1 ml of the relevant pretreated sample solution was added to training and prediction set. The concentration of caffeine was varied between 8 and 25 μg ml^{-1}, theobromine and theophylline between 1 and 10 μg ml^{-1} in synthetic mixtures. Composition of validation set is shown in Table 3.

3.2. Wavelength selection

Multivariate calibration methods have generally been considered as full-spectrum in the processes of calibration and prediction. But, most often, selecting the undesired regions of the spectrum evaluated is provided more noise in the analytical results (Brown, 1992). Rossi and Pardue (1985) showed that accuracy can be improved by careful wavelength selection. So far, various criteria have been developed to allow for wavelength selection. Alba, Martinez, and Rodriguez (2002) proposed a simple and new method for feature selection. In this method, wavelength selection was performed by selecting the region that independent and dependent variables high correlated with each other.

In the present work, the UV-spectra of caffeine, theobromine and theophylline solutions were recorded, in the wavelength range of 200–350 nm with 1 nm interval

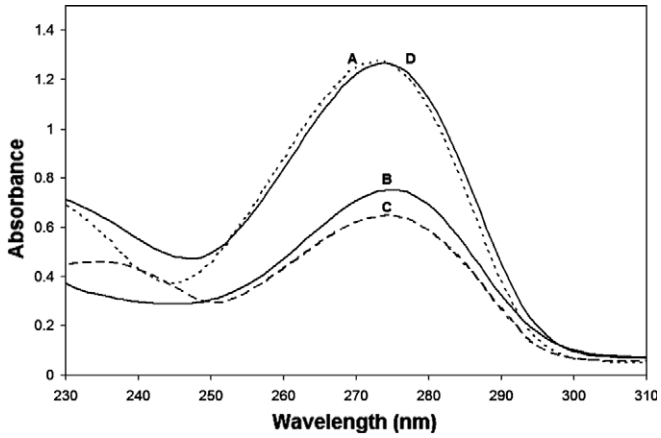

Fig. 1. Absorption spectra of 25 μg ml^{-1} caffeine (A), 10 μg ml^{-1} theophylline (B), 10 μg ml^{-1} theobromine (C) and mixture of caffeine (11 μg ml^{-1}), theophylline (5 μg ml^{-1}) and theobromine (6 μg ml^{-1}) (D).

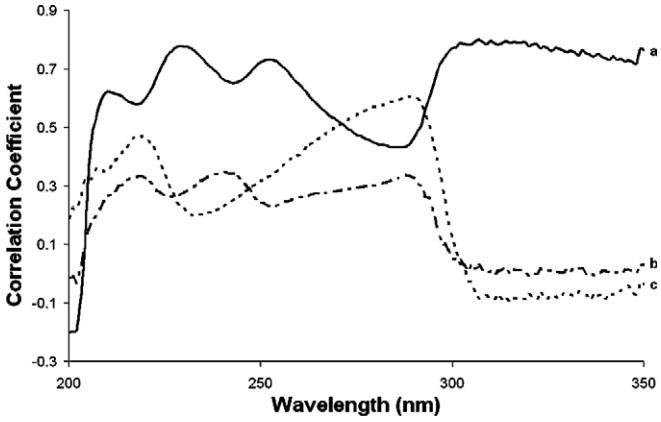

Fig. 2. Plot of correlation coefficient vs. wavelength for caffeine (a), theobromine (b) and theophylline (c).

(Fig. 1). Correlation coefficients between concentration of each component in calibration set and absorbance at different wavelengths was calculated and this relation is shown in Fig. 2. For exact selection of the wavelength regions, the values of root mean square difference (RMSD) and relative error of prediction (REP) were calculated for four spectral regions and demonstrated in Table 1. The results show that the 230–300 nm spectral regions with 71 data point have high correlation coefficients and minimum RMSD and REP compared with the others. For this reason the analysis of real samples was achieved in this limit.

3.3. Modeling with PLS and PC-ANN

A calibration matrix with dimension of 71×25 was used for training in PLS algorithm. The minimum PRESS was found at 23 latent variables (LVs) (Fig. 3), but 6 LVs were selected because of three reasons: small difference between PRESS at 6 and 23 LVs, for avoidance of complexity of the model and for avoidance of over-fitting.

After principle component analysis on data matrix, 5 PCs was selected and the scores on the selected PCs were subjected to ANN as input. The loadings were used for transformation of prediction set and real samples to new space of the five PCs. A 5×25 data matrix of scores was used for training of ANN. Network weights initially assigned random values between +1 and −1. A linear transfer function was used between both input-hidden and hidden-output layers. The network gave the best performance with five neurons in hidden layer. Because there are three components in mixtures, the output layer contained three neurons.

3.4. Statistical assessments of the models

After modeling with PLS and PC-ANN, the qualities of the models were assessed by prediction on 5 samples vali-

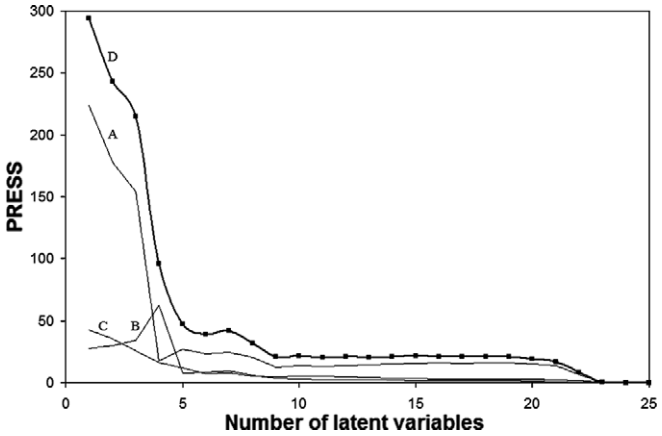

Fig. 3. PRESS curve for PLS modeling. Caffeine (A), theobromine (B), theophylline (C) and for all components (D).

Table 1
Effect of the spectral region on predictive ability of PLS and ANN models

Spectral region (nm)	PLS						ANN					
	RMSD			REP$_s$ (%)			RMSD			REP$_s$ (%)		
	CF	TB	Total	CF	TB	Total	CF	TB	Total	CF	TB	Total
210–300	0.49	0.19	0.37	3.42	7.61	5.78	0.47	0.37	0.42	3.30	14.86	6.66
210–290	0.38	0.31	0.33	2.71	12.48	5.17	0.40	0.24	0.32	2.82	9.83	5.06
230–300	0.46	0.11	0.31	3.22	4.73	4.97	0.45	0.16	0.31	3.16	6.36	4.86
250–300	0.47	0.51	0.42	3.32	20.51	6.64	0.48	0.43	0.41	0.41	17.30	6.35

Table 2
Amount of caffeine and theobromine in real samples analysed by HPLC and predicted values by PC-ANN and PLS

Sample	PC-ANN[a]		PLS[b]		HPLC	
	CF/mg g^{-1}	TB/mg g^{-1}	CF/mg g^{-1}	TB/mg g^{-1}	CF/mg g^{-1}	TB/mg g^{-1}
Otaghvar–Lahijan (Black tea)	16.20	0.44	17.11	0.40	19.60	0.41
Otaghvar–Lahijan (Green tea)	16.05	0.44	16.01	0.5	17.61	0.34
Divashal–Langrood (Black tea)	12.23	0.39	11.29	0.44	14.53	0.36
Ghalami–Lahijan (Black tea)	10.80	0.57	9.94	0.52	12.32	0.45

All values calculated as milligram of component per gram of tea leave.
[a] A 5-5-3 feed-forward artificial neural network.
[b] PLS model was constructed with 6 LVs.

dation set (Table 3), results are shown in Table 4. The correlation plots for each component are shown in Fig. 4.

The REP for each component (REP_s) and all components (REP_t) were used for descriptions of predictive ability of the models (Otto & Wegscheider, 1985). REP_s for component j is defined as:

$$REP_{s_j} = \frac{100}{\bar{x}_j}\sqrt{\frac{1}{n}\sum_{i=1}^{n}(\hat{x}_{i,j} - x_{i,j})^2}$$

and REP_t is defined as:

$$REP_t = \frac{100}{\bar{x}}\sqrt{\frac{1}{N}\sum_{j=1}^{m}\sum_{i=1}^{n}(\hat{x}_{i,j} - x_{i,j})^2}$$

where $\hat{x}_{i,j}$ is the estimated concentration of component j in sample i, and $x_{i,j}$ is the actual concentration of this component. n is the number of samples in validation set and m is the number of components. N is the product of n and m. $\bar{x}_j$ is the mean of concentration of component j in all samples and $\bar{x}$ is the mean of all concentrations in validation set.

Table 3
Composition of validation set for both PLS and PC-ANN

Sample	CF/μg ml^{-1}	TB/μg ml^{-1}	TP/μg ml^{-1}
1	9.2	4.5	3.0
2	11.2	2.0	1.0
3	13.2	1.0	4.0
4	16.4	3.5	3.0
5	21.4	1.5	1.0

3.5. Real sample analysis

Tea samples were prepared according to Section 2.4, and samples were analyzed using HPLC. Retention times were approximately 2.18, 3.36 and 5.94 min for theobromine, theophylline and caffeine, respectively. Table 2 shows results of HPLC analysis.

From the HPLC analysis it was found that caffeine was high (compared to theobromine) and theophylline was not detected in all tea samples. Tea sample solutions were diluted so that the caffeine concentration was within the calibration range. Due to low theobromine level in resultant solutions, these solutions were spiked with theobromine so that the final concentrations were within the calibration range of each component. Prediction results of these real samples were calculated after removing spiked value contribution and are shown in Table 2.

Schulz, Engelhardt, Wegent, Drews, and Lapczynski (1999) analyzed 95 tea samples (43 samples have been collected from Thailand and 52 samples have been supplied by various German tea packers) and showed that the amount of caffeine and theobromine in tea leaves varied from 3.3 to

Table 4
Prediction results of validation set

Sample number	PC-ANN[a]			PLS[b]		
	CF/μg ml^{-1}	TB/μg ml^{-1}	TP/μg ml^{-1}	CF/μg ml^{-1}	TB/μg ml^{-1}	TP/μg ml^{-1}
1	8.7	4.2	2.5	8.5	4.3	2.5
2	11.7	1.9	1.1	11.7	1.9	1.1
3	13.2	0.9	3.9	12.8	1.0	3.8
4	16.6	3.5	2.8	16.8	3.5	2.9
5	21.9	1.6	1.1	21.7	1.6	1.1
m[c]	1.06	0.95	0.88	1.06	0.94	0.88
b[d]	−0.66	0.05	0.18	−0.84	0.09	0.14
R[e]	0.9976	0.9976	0.9865	0.9963	0.9973	0.9890

[a] A 5-5-3 feed-forward artificial neural network.
[b] PLS model was constructed with 6 LVs.
[c] Slopes for regression equations of predicted and actual value of each component.
[d] Intercepts for regression equations of predicted and actual value of each component.
[e] Correlation coefficients for regression equations of predicted and actual value of each component.

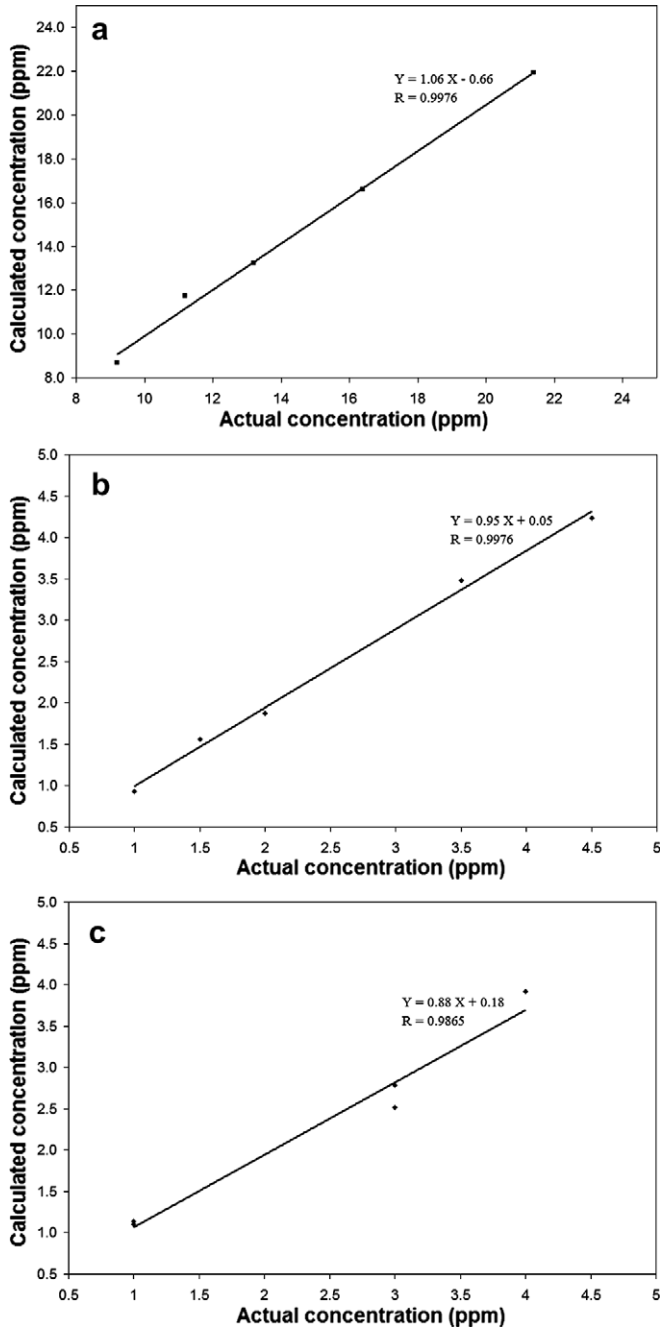

Fig. 4. Correlation plots of the calculated concentrations with PC-ANN model vs. actual concentrations for validation set. Caffeine (a), theobromine (b) and theophylline (c).

50.5 mg/g (mean 34.9 mg/g) and 0.2 to 4 mg/g (mean 1.4 mg/g), respectively. The content of caffeine and theobromine in iranian tea are within this range but lower than mean.

4. Conclusions

Spectrophotometry was coupled with PLS and ANN for determination of caffeine and theobromine in tea samples. REP_s on synthetic samples showed that results of two modeling methods are comparable. Prediction performance of both models on theobromine was poorer than caffeine, because of very low concentration of this component in samples. A good correlation between predicted values and HPLC results was obtained for real samples. Finally, PLS and ANNs with spectrophotometric measurements can be used as a simple and fast method with acceptable results for determination of caffeine and theobromine in tea samples.

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