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Total Polyphenols in Green Tea Samples by FT-NIR Spectroscopy

Vadakkepulppara Ramachandran Nair Sinija* • **Hari Niwas Mishra****

Agricultural & Food Engineering Department, Indian Institute of Technology, Kharagpur, West Bengal-721 302, India

Corresponding authors: * **sinijavr@rediffmail.com** ** **hnm@agfe.iitkgp.ernet.in**

ABSTRACT

The feasibility of measuring total polyphenols content in instant green tea powder and green tea granules was investigated by Fourier Transform Near-Infrared (FT-NIR) spectroscopy. The spectra were measured in diffused reflectance mode by keeping 8-10 g samples in a small sample bottle. A partial least square regression model was developed with vector normalization as the pre-processing method in the NIR region (4000-12000 cm^{-1} or 800-2500 nm). The developed model was validated using a cross validation technique. FT-NIR spectroscopy with chemometrics, using PLS – vector normalization as the pre-processing method – could predict the total polyphenols content in tea samples in terms of gallic acid accurately up to an R^2 value of 0.978 and a standard error of cross validation (RMSECV) value of 1.45 with 4 factors in the prediction model. The developed model was applied to predict total polyphenols in green tea samples within 30-60 min. The developed procedure was further validated with fresh samples which were not used for calibration and compared with spectroscopic method of ploy phenol determination. The overall results demonstrate that NIR spectroscopy with multivariate calibration could be successfully applied as a rapid method not only to identify tea varieties but also to determine total polyphenols content in green tea samples.

Keywords: chemometric analysis, Fourier transform near infrared spectroscopy, instant tea, PLS regression model, spectral pre-processing

INTRODUCTION

Tea (*Camellia sinensis*) is the second most consumed beverage in the world after water (Li and He 2008). With the increasing consumption of tea, its quality control has becomes more and more important nowadays, for example, many national and international authorities are setting criteria for quality factors like total polyphenols and caffeine (Airy 1999; Chen *et al*. 2006). In general, caffeine and total polyphenols are analyzed as the important quality factors for tea leaves. These constituents (polyphenols and caffeine) are mainly responsible for the characteristic astringent and bitter taste of tea brews (Zhang *et al.* 1992). Additionally, most commercially tea leaves have many varieties on the market, which differ not only from a botanical standpoint but also in terms of quality. These differences are recognized commercially and also appreciated by consumers.

In the past few years, many different methods of analysis have been employed to identify tea varieties and to determine the chemical composition of tea. Some approaches were applied to identify tea varieties using modern techniques like high-performance liquid chromatography (HPLC), gas chromatography (GC), plasma atomic emission spectrometry, FTIR-ATRI, Raman Spectroscopy, CE-MS/MS, LCMS, etc., (Togari *et al*. 1995; Horie and Kohata 2000; Zuo *et al.* 2002; Santose *et al*. 2005; Sawalha *et al*. 2009). Also some approaches were applied to quantitatively analyse the chemical composition of tea leaves, such as HPLC (Zuo *et al.* 2002), capillary electrophoresis (Horie *et al.* 1997), colorimetric measurements (Chen *et al*. 2009) and titration with potassium permanganate (ISO 1994). However, all of the methods mentioned above are time-consuming.

Near infrared (NIR) spectroscopy has proved to be a powerful analytical tool used in the agricultural, nutritional, petrochemical, textile and pharmaceutical industries (McGlone *et al*. 2002; Diez 2004; Woodcock *et al.* 2008; Fernández-Ibañez *et al.* 2009). Since the 1990s, attempts have been made to simultaneously predict water, alkaloid and phenolic substance content in tea leaves using NIR spectroscopy (Hall *et al.* 1988; Schulz *et al.* 1999). Studies on the application of NIR spectroscopy to quantitative analysis of total antioxidant capacity in green tea was also reported (Lupaert *et al.* 2003; Zhang *et al.* 2004). Although they provided some better results (more sensitive and rapid) for tea using NIR spectroscopy, they did not discuss details of the prediction models even without using independent test samples to test the robustness of the models, such as Schulz *et al.* (1999).

Green tea contains polyphenols, which include flavanols, flavandiols, flavonoids, and phenolic acids; these compounds may account for up to 30% of the dry weight. Most of the green tea polyphenols are flavonols, commonly known as catechins. Tea polyphenols are of great interest due to their beneficial medicinal properties (Yang *et al*. 2002). There is increasing evidence that polyphenols substances found in tea can enhance general health. Recently, many researches have suggested that antioxidants found in polyphenolic substances may play an important role in the prevention of cardiovascular disease (Nakachi *et al.* 2000), chronic gastritis (Shibata *et al.* 2000; Setiawan *et al.* 2001) and some cancers (Fujiki *et al*. 2001; Inoue *et al*. 2001; Jian *et al.* 2004). Additionally, polyphenolic compounds are mainly responsible for the characteristic astringent and bitter taste of tea brews. In recent years, many methods of analysis have been employed to determine total polyphenols content in tea, such as colorimetric measurements and the titration method with potassium permanganate (ISO 1994). However, these methods are all time-consuming. NIR spectroscopy is a fast, accurate and non-destructive technique that can be employed as a replacement of time-consuming chemical methods. In all other chemical and analytical

Received: 12 January, 2009. Accepted: 21 May, 2009.

Original Research Paper

methods sample preparation is needed and it is destructive also. Time taken for individual measurement is very long. In FT NIR once the calibration is over, it will take only seconds to determine the amount of a particular component in the sample without any sample preparation.

The objective of present study was to develop a rapid method for the quantitative estimation of total polyphenols content in instant green tea powder and green tea granules using FT-NIR spectroscopy. In commercial production, we can use this method to analyze the amount of total polyphenols in the final product and also to check the same during packaging and storage as a very rapid and non-destructive method. The monitoring and determination of polyphenols concentration in tea products is essential for manufacturers because most of the beneficial effects of tea are mainly due to the polyphenols.

MATERIALS AND METHODS

Sample preparation

The fresh tea leaves for preparation of tea samples were plucked from the tea gardens of the Indian Institute of Technology, Kharagpur, India. Fresh tea leaves were steamed (1 kg/cm^2 for 1-2 min) immediately after plucking to arrest the fermentation process (oxidation reaction) and then ground in a laboratory grinder. From the paste thus obtained, a portion of the juice (40-45%) was extracted by means of hydraulic pressing in an extraction unit developed at the Agricultural & Food Engineering Department of IIT Kharagpur, India (Sinija and Mishra 2009). The juice with a total solid content of 6-9% was used to produce instant tea powder samples by freeze drying and a pressed leaf residue with a moisture content of 63-65% wet basis (wb) was subjected to hot air drying (temperature: 45-55°C, thickness: 3-5 mm, air velocity: 1.5-2.5 m/s) in a recirculatory convective air drier (Bose Instruments Pvt. Ltd., Kolkata, India) to produce different green tea granules samples. Since different drying conditions were employed, samples with different amounts of total polyphenols thus resulted.

An FT-NIR MPA^{TM} spectrometer (Bruker Optics, Germany) combined with Opus 5.5 software was used for analysis of tea samples by generating a unique spectrum for each sample. This spectrometer with an integrated Michelson interferometer utilized the Fourier-Transform and had distinct advantages compared to dispersive spectrometers. The Michelson interferometer in FTNIR has two basic advantages. First, the Fellgett multiplex advantage derives from the simultaneous processing of the entire spectral range during a single scan. All frequencies in the spectra are measured simultaneously in Fourier transform near-infrared (FTNIR) spectrometer for the entire time. This is because an interferometer can modulate at frequencies that are proportional to the wave length. The time advantage is even larger, since it is directly proportional to spatial elements examined. A complete spectrum can be collected very rapidly, and many scans can be averaged in the time taken for a single scan of a dispersive spectrometer. Second, the Jacquinot advantage allows for a large energy throughput because it is possible to use a large aperture. For the same resolution, the energy throughput in an FTNIR spectrometer can be higher than a dispersive spectrometer, where it is restricted by the slits. In combination with the multiplex advantage, this leads to one of the most important features of a FTNIR spectrometer: the ability to achieve the same signal-to-noise ratio as a dispersive instrument in a much shorter time. Also, the intrinsic wavelength scale in a FTNIR spectrometer provides wavelength repeatability better than one part in a million (Connes advantage). The wave number calibration of interferometers is also much more precise (Brain 2001; William and Norris 2001).

Chemometrics: multivariate analysis

Multivariate analysis was used for quantitative and qualitative analysis. A Partial Least Square (PLS) algorithm, which was proven to be effective in many quantitative applications (determination of total solids, pH, caffeine in tea (Chen *et al.* 2006)), was used in the present study. The OPUS 5.5 software was used for PLS analysis. These methods with original and vector normalised spectra were used to develop calibration models. The performance of the final PLS model was evaluated in terms of root mean square error of cross-validation (RMSECV) for cross validation and root mean square error of prediction (RMSEP) during test validation, and the coefficient of determination (R^2). For RMSECV, a leave-one-sample-out cross-validation was performed: the spectrum of one sample of the training set was deleted from this set and a PLS model was built with the remaining spectra of the training set. The left-out sample was predicted with this model and the procedure was repeated by leaving out each of the samples of the training set. In test validation a set of samples were identified as test data and with the remaining data set a calibration model was developed first and then the test spectra was used for the validation of the developed model (Sinija and Mishra 2009).

The number of PLS vectors used is defined in the OPUS software by the size of the "rank". The optimum PLS rank can be calculated only if the number of calibration spectra is sufficiently high (e.g. one component and 20 calibration spectra). The PLS regression has the advantage that the PLS factors are arranged in correct sequence, according to their relevance to predict the component values. The first factor explains the most drastic changes of the spectrum.

The residual (Res) is the difference between the true and fitted value. Thus the sum of squared errors (SEE) is the quadratic summation of these values (Eq. 1).

$$SSE = \sum [\mathrm{Re}\, s_i]^2 \quad \text{.....(1)}$$

The root mean square error of estimation (RMSEE) is calculated from this sum, with "n" being the number of samples and "r" the rank (Eq. 2).

$$RMSEE = \sqrt{\frac{1}{n-r-1}} \times SSE \quad \text{.....(2)}$$

The determination coefficient, R^2 (Eq. 3) gives the percentage of variance present in the true component values, which is reproduced in the regression. R^2 approaches 100% as the fitted concentration values approach the true values.

$$R^2 = \left(1 - \frac{SSE}{\sum (y_i - y_m)^2}\right) \times 100 \quad \text{.....(3)}$$

where y_m is the mean of the reference results for all samples. R^2 can be negative (in some cases) for low ranks, when the residual are larger than the variance in the true values (y_i). In case of cross validation the RMSECV is calculated using Eq. 4.

$$RMSECV = \sqrt{\frac{\sum_{i=1}^{n} (\bar{y}_i - y_i)^2}{n}} \quad \text{.....(4)}$$

where n is the number of samples in the training set, y_i the reference measurement result for the sample i and $\bar{y}_i$ is the estimated result for sample i when the model is constructed with the sample i removed. The number of PLS factors included in the model is chosen according to the lowest RMSECV. This procedure is repeated for each of the pre-processed spectra.

For the test set, the RMSEP is calculated as follows (Eq. 5).

$$RMSEP = \sqrt{\frac{\sum_{i=1}^{n} (y_i - \hat{y}_i)^2}{n}} \quad \text{.....(5)}$$

where, n is the number of samples in the test set, yi the reference measurement result for test set sample i and $\hat{y}_i$ is the estimated result of the model for test sample i.

Preparation of calibration and validation models

Spectra of 30 different green tea samples (with different polyphenol contents, 14-29%) prepared were used for model development. The samples were prepared with different raw materials (different

variety and different plucking levels) and varying processing conditions in order to have different polyphenol content. The NIR spectra were collected in the reflectance mode using the FT-NIR MPA^{TM} spectrometer. The range of spectra was from 12,000 to 4000 cm^{-1}. The standard sample accessory holder was used for performing the tea spectra collection. For each tea sample, 8-10 g of dry tea leaves was filled into the sample cup in the standard procedure depending upon the bulk density of materials. The corresponding amount of dry tea powders was densely packed into the sample cup and then compressed by closing it. For each sample, three spectra were recorded at three different points by rotating the sample bottle by 120°. The average of the three spectra, which were collected from the same tea sample, was used in the next analysis. The temperature was kept around 25°C and the humidity was kept at a steady level in the laboratory. The amount of total polyphenols in each sample was determined by the standard method using Folin Ciocalteau reagent which is described below. Cross validation method was used in this model.

Estimation of total polyphenols by spectrophotometric method

Five grams of green tea sample was ground well in a mortar and pestle with 100 ml of 95% ethanol and the volume made up to 250 ml. Two ml of this extract was diluted to 100 ml with distilled water. In a boiling test tube 2 ml of the diluted extract, 4 ml of Follin Ciocalteau (FC) reagent and 2 ml of sodium carbonate solutions were added, diluted to 12 ml with distilled water and shaken well. This mixture was allowed to stand for 30 min at room temperature. After that the blue colour developed in the sample at 760 nm was read by a spectrophotometer at room temperature. Phenols react with phosphomolybdic acid in FC reagent in alkaline medium and produce a blue colored complex. A standard curve was prepared with gallic acid for total polyphenol estimation in the concentration range between 0 and 30% (Sadasivam and Manickam 1996).

Validation using fresh samples

Polyphenol content of fresh green tea sample (any sample with polyphenol content different from the set used for calibration, but within the range used for calibration) was determined using the spectrophotometric method in order to verify the accuracy of the developed method. Triplicate measurements were taken for the same sample and results were analyzed statistically. One-way ANOVA was carried out for the result obtained by both the methods and p value was calculated, from which it is clear whether there is significant difference between the results obtained by two methods. Triplicate measurements were taken for the same sample and the average value was used for comparison.

RESULTS AND DISCUSSION

Fig. 1 shows the FT-NIR spectra of tea samples used for developing the calibration model. These true peaks were selected after smoothing the spectrum to avoid interference due to noise. The most intensive band in the spectrum belongs to the vibration of the second overtone of the carbonyl group (5285 cm^{-1}), followed by the -CH (7137 cm^{-1}), the -CH_2 (5472 cm^{-1}) and the -CH_3 overtone (5808 cm^{-1}). The vibration of the C=O, -CH and -CH_2 are caused by ingredients such as polyphenols, alkaloids, protein, volatile as well as non-volatile acids and by some aroma compounds (Paradkar and Irudayaraj 2002).

The NIR region contains bands that often overlap, making it difficult to extract spectral parameters of the individual bands. Chemometrics have provided a way of overcoming these problems through empirical models that relate the multiple spectral intensities from many calibration samples to known analytes in these samples. As the spectra show similar basic FTNIR spectral patterns, mathematical transformations were required to use the FTNIR data for quantitative analysis. Despite the lack of distinct peaks, it has been shown the PLS can extract relevant information for quantitative determinations (McShane and Cote 1998).

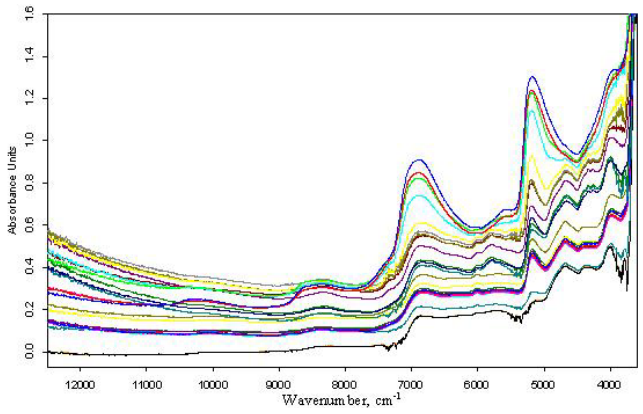

Fig. 1 FT-NIR absorption spectra of calibration data set. Different colours correspond to the different samples (30 samples used for this study).

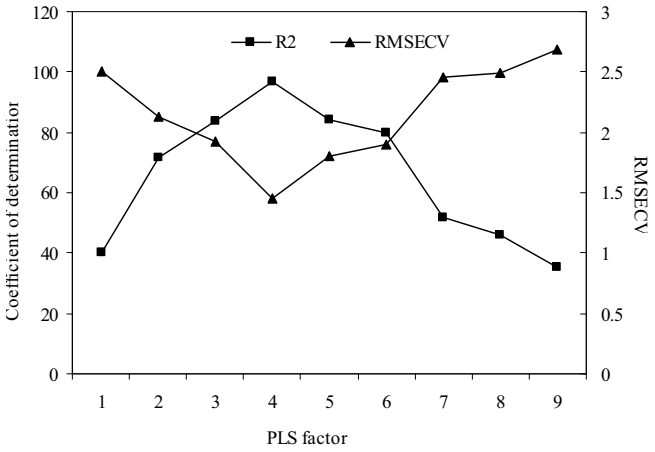

Fig. 2 RMSECV and r^2 plotted as a function of PLS factors.

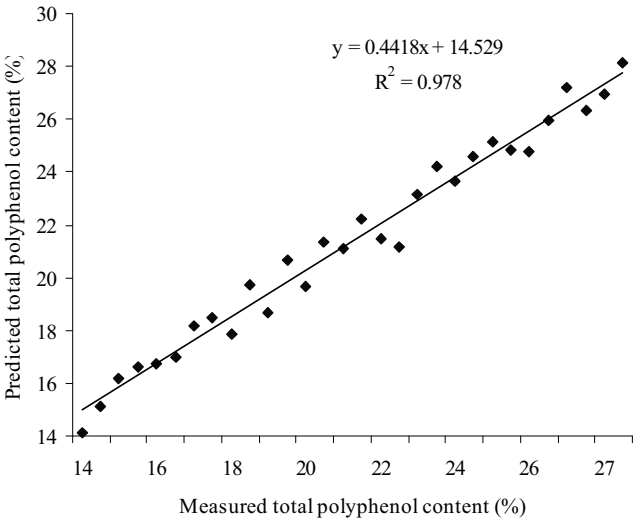

Fig. 3 Linear regression plot of measured versus predicted content of total polyphenols.

In the application of PLS algorithm, it is generally known that spectral preprocessing methods and the number of PLS factors are critical parameters. Here their effects on the results are discussed. The optimum number of factors is determined by the lowest RMSECV and highest value for r^2. **Table 1** shows the values of r^2 and RMSECV for different pre-processing techniques used. From the table it is clear that compared with others, the lowest RMSECV value equals 1.45 obtained after the vector normalization spectral pre-processing and also maximum value for coefficient of determination. **Fig. 2** shows values of RMSECV and R^2 cor-

Table 1 RMSEP and R^2 values corresponding to each PLS factor for determining caffeine content with different spectral pre-processing methods.

Pre-processing technique	R^2 (validation)	RMSECV	R^2 (calibration)	RMSEE	PLS factor
No pre-processing	94.23	4.01	96.58	2.16	7
Vector normalization	97.80	1.45	98.03	1.81	4
1st derivative	84.38	9.70	91.66	7.03	4
Straight line subtraction	90.30	7.44	95.89	6.62	5
2nd derivative	98.36	2.08	99.78	1.81	8

Table 2 Comparison of results obtained by FTNIR method and spectroscopic method for total polyphenol determination.

Summary						
Groups	*Count*	*Sum*	*Average*	*Variance*		
Spectrometric method	3	65.38	21.79333	0.430033		
FT-NIR method	3	68.05	22.68333	0.143233		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.18815	1	1.18815	4.145191	0.111453^{NS}	7.708647
Within Groups	1.146533	4	0.286633			
Total	2.334683	5				

NS – non significant

responding to each PLS factor for determining total polyphenols with vector normalization as the spectral pre-processing method. Seen from figure it is clear that the maximum value of R^2 and minimum RMSECV value is for PLS factor 4 in vector normalization method. This model needs four PLS factors. In this application, vector normalization seems to perform better than other pre-processes.

The NIR predicted results for total polyphenol content in green tea samples using the calibration model is presented in **Fig. 3**. The PLS-regression method gave R^2 values of 0.978 for calibration data set and RMSECV value of 1.45 for cross validation. The results of this study clearly demonstrated the capability of FTNIR for this application. The predictive abilities of the calibration models were evaluated by the residual predictive deviation (RPD) also, which was defined as the ratio of the standard deviation of the reference data to the standard error of predicted data for the population tested (Pink *et al*. 1998). RPD value for the present model is 4.5, which is greater than three and considered to be desirable for prediction purposes as per the previous references (Pink *et al*. 1998; Nieuwoudt *et al.* 2006).

Analysis of tea samples

The result obtained by the developed method was validated with fresh tea sample and cross checked by standard spectrophotometric method. Total polyphenol content of freshly prepared tea samples were measured using the above two methods. Total polyphenol content values for each sample was measured in triplicate by the above 2 methods and the values obtained were analyzed statistically. The results of the ANOVA (**Table 2**) shows that $F_{critical}$ is greater than $F_{tabulated}$ and that the p value is 0.1114, which shows that there is no significant difference between the values obtained by the two methods even at 5% level of significance. This proved that FTNIR spectroscopy is a rapid and efficient tool for detection and quantification of moisture content in green tea samples.

CONCLUSIONS

A rapid and simple FT-NIR procedure to estimate the amount of total polyphenols in green tea samples was developed using a single calibration model. The model was developed using the spectral region 4000-12000 cm^{-1}. The maximum coefficient of determination (R^2) value of 0.978 and RMSECV value of 1.45 was obtained for model developed for prediction of moisture content. The performance of developed method was confirmed with freshly prepared samples and the values obtained for total polyphenol content of samples by this method was found to have no significant difference with the values obtained by spectrophotometric method. Time taken for individual measurement is between 30-60 seconds. This method can be adopted directly by the industries in order to determine the total polyphenolic content of green tea samples at various stages of processing to check the quality and also at the time of packaging or during storage without destroying the sample.

ACKNOWLEDGEMENT

The first author is thankful to All India Council for Technical Education (AICTE) New Delhi, for the financial assistance received in the form of National Doctoral Fellowship to pursue her Doctoral programme.

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