The Journal of Applied Sciences Research

Journal homepage: http://www.journals.wsrpublishing.com/index.php/tjasr

Online ISSN: 2383-2215 Print ISSN: 2423-4400

Original Article

Rapid Evaluation of Antioxidant activity of freeze dried Lemon juices by Fourier Transform Infrared Spectroscopy and Chemometric Analysis

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ABSTRACT

The prospect of using Fourier transform infrared (FTIR) spectroscopy in combination with multivariate analysis as a prompt quantitative tool to evaluate the antioxidant activity (AA) of lemon juice extracts was investigated. Partial least square (PLS) and principal component regression (PCR) were used and optimized to develop the calibration models using spectrum regions ranging from 1900 cm^{-1} to 950 cm^{-1}. The evaluation of developed models was carried out by cross-validation in order to find out the standard error of models including root mean square error of cross validation (RMSECV), root mean square error of calibration (RMSEC) and root mean square error of prediction (RMSEP). The best model used to evaluate the antioxidant activity was obtained by PLS algorithm with coefficients of determination (R^2) of 0.99864 and

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How to Cite this Article: Bhutto, A.A., Sherazi, S.T.H., & Mahesar, S.A. (2018). Rapid Evaluation of Antioxidant activity of freeze dried Lemon juices by Fourier Transform Infrared Spectroscopy and Chemometric Analysis. *The Journal of Applied Sciences Research*, 5(1):14-26.
Retrieved from http://www.journals.wsrpublishing.com/index.php/tjasr/article/view/438

Article History: Received: 2018-09-20 Accepted: 2018-10-02

RMSEC of 0.171. Comparison of predicted FTIR results with standard DDPH (1,1-diphenyl-2- picrylhydrazyl) assay validated that FTIR spectroscopy combined with multivariate analysis can be efficiently used to evaluate the antioxidant potential of lemon juices.

Keywords: Antioxidant activity, DPPH radical scavenging assay, Fourier transform infrared spectroscopy, Partial least square (PLS), Principal component regression (PCR).

INTRODUCTION

Plant materials including fruits and vegetables are largely characterized by antioxidant activity (Arnao, 2000), which is attributed to compounds that are responsible to protect the biological system from the harmful effects of reactions including the reactive oxygen species (ROS) and reactive nitrogen species (RNS). Such protective abilities of antioxidant compounds are of increased instrest within biological, medical, and nutritional fields (Karadag *et al.,* 2009). Fruits largely contribute the antioxidants in the diet, mostly because of presence of vitamins, phenolic compounds and carotenoids (Leopold *et al.,* 2012). Lemons are important part of fruits, specially for their role in prevention of diseases including obesity, blood lipid lowering, diabetes, cardiovascular diseases and some types of cancer (González-Molina *et al.,* 2010).

Deterioration of significant proportion of fruits and foods antioxidants takes place during preliminary processing, preservation and prolonged storages. Inappropriate drying processes, particularly involving temperature, can cause the loss of nutritional value (Korus, 2011). Dehydration is most commonly used method for fruits and vegetables preservation aiming the removal of water to the level so that microbial spoilage and deterioration reactions are inhibited (Scala and Crapiste, 2008). Freeze-drying is relatively a better procedure used for removal of water and to get a final products of highest quality. This method causes dehydration of the frozen samples by sublimation. In the absence of moisture content and the low temperatures required for the procedure, most microbiological reactions are inhibited, giving a final product of excellent quality with increased antioxidant activity (Korus, 2011).

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay is one of the frequently used method for in vitro assessment of antioxidant capacity of fruits, foods and other plant materials (Bunaciu *et al.,* 2012). Antioxidant activity obtained by single assay cannot be precisely reported, hence it is recommended that at least two (or even all) of assays be combined to obtain a precise picture of the total antioxidant activity of the foods and fruits (Contreras-Calderóna *et al.,* 2011). Many methods used for evaluation of antioxidant capacity suffers from the limitations including assessment of hydrophilic antioxidants, the reaction end point determination problems, the analysis in physiological irrelevant pH, potential interference from some food components as well as the use of various standards causes the complexities in expression of results hence no method is supposed to be most convenient and a standard method for precise assessment of antioxidant capacity, resulting in the requirement of reliable, simple and convenient method for evaluation of antioxidant activity (Karadag *et al.,* 2009).

FTIR (Fourier transform infrared spectroscopy) combined with chemometric methods is an excellent analytical tool and interesting research methodology, as it has already shown to be a means of rapid antioxidant evaluation of different fruits (Lam *et al.,* 2005; Leopold *et al.,* 2012), foods (Lu *et al.,* 2011; Lu and Rasco, 2012) and medicinal plants (Bunaciu *et al.,* 2012). ATR (Attenuated total reflectance) is most commonly used technique in mid-IR region that provides an easy approach by eliminating many sample handling problems and finds applications in many areas, including food samples (Sherazi *et al.,* 2009).

In this study, FTIR spectroscopy and chemometric methods are combined to develop a fast, uncomplicated, reliable and cost effective method for evaluation of antioxidant capacity of freeze dried lemon juices. The current developed method could be used as an alternative quantitative method for assessing the quality of fruits and foods products and by-products.

MATERIAL AND METHODS

Plant materials and sample preparation

Lemon fruits (*Citrus limon*) were collected from local market of Hyderabad, Pakistan during June 2017. Juice was mechanically extracted and freeze dried using Labconco Freeze zone 4.5. Freeze drying was carried out at -40 ^{0}C until cohesive and physically stable mass was obtained. Freeze-dried samples were stored at 4 ^{o}C in an air-tight container for further analysis.

Chemicals

Methanol (HPLC-grade) and HCl (ACS-grade) were purchased from Fisher Scientific Chemicals (USA) while 2,2-diphenyl-1-picrylhydrazyl (DPPH) was supplied by Sigma-Aldrich. All the reagents and chemicals used were of analytical grade.

Sample extraction

5 grams of freeze dried samples were extracted in triplicate with 25 mL of 80 % methanol in an ultrasonic bath at ambient temperature for 20 min. The extracts were filtered and freeze dried. The dry extracts were stored at -20 ^{o}C prior to analysis.

Free radical scavenging activity

Free radical scavenging activity of lemon juice extracts was carried out by using 2,2´-diphenyl-1-picrylhydrazyl radical (DPPH·) scavenging assay reported by Leong and Shui (2002). Briefly, 0.2 mL of diluted juice extracts were added in test tubes containing 3.0 mL of freshly prepared DPPH solution of 100 µM concentration. Test tubes were incubated at room temperature for 30 min followed by vigorous shaking. Control was prepared by same procedure but without samples. Methanol was used as a blank and decrease in absorbance was recorded at 515. Measurements were performed in triplicate and percentage of DPPH$^{\cdot}$ scavenged (% DPPH$^{\cdot}_{sc}$) was determined using equation:

$$\text{Scavenging activity (\%)} = (A_{control} - A_{sample}) \times 100 / A_{control}$$

Where $A_{control}$ is the absorbance of control, prepared in the absence of samples and A_{sample} is the absorbance of sample. The fresh prepared radical was used and absorbance was recorded to check the stability of the radical during the time of analysis.

FT-IR spectral measurements

Fourier transform infrared (FTIR) spectrometer (Thermo Nicolet 5700 FTIR, Madison, WI) supplied with deuterated triglycine sulfate (DTGS) detector and operated by OMNIC software (version 7.2) was employed to record the Infrared spectra of freeze dried lemon juice extracts. The sampling compartment of the instrument contained the attenuated total reflectance accessory with a removable zinc selenide (ZnSe) crystal. Freeze dried lemon juice extract was directly placed onto the crystal and a reasonable contact between the crystal and sample was achieved by pressing the crystal with a press accessory. The IR spectrum of each extract was collected in triplicate at room temperature, against a background of air, over the wavenumber range of 4000–600cm^{-1}, with an average of 32 scans at a resolution of 4 cm^{-1}. An average spectrum was obtained from the triplicates of each sample extract. After each analysis, the ZnSe crystal was carefully washed with methanol to eradicate any deposits and allowed to dry at room temperature (Mahesar *et al.,* 2011).

Multivariate analysis

Turbo Quant analyst software package (TQ 7.2, Nicolet, Madison, WI) was used to determine the relationship between each FTIR sample spectrum and its antioxidant activity. Partial least square (PLS) and principal component regression (PCR) models were developed, which are most frequently used multivariate calibration methods in chemometrics. Twenty one averaged FTIR lemon juice extract spectra were used to build a regression model. The cross-validation, using leave-one-out method, was used for determination of the maximum number of significant factors (PCs) in order to confirm the predictive capability and to avoid over-fitting of the data (Versari *et al.,* 2010).

RESULTS AND DISCUSSION

Antioxidant activity

The stable DPPH radical is reduced in radical scavenging assay, resulting the decrease in absorbance measured at 515 nm. DPPH radical scavenging activity in lemon juice samples ranged from 47.73 ± 0.93 to 59.61 ± 0.62 (percent inhibition) as shown in Figure 1.

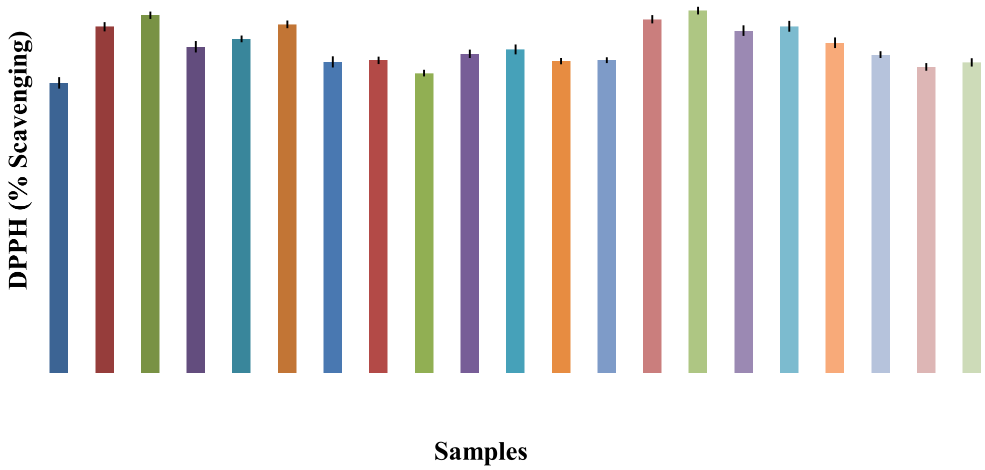

Figure 1: DPPH Scavenging activity of lemon juice samples. Values represents means of triplicate analysis, bars show ± SD

DPPH percentage inhibition of freeze dried lemon juices was observed relatively higher than those reported by Xu *et al.,* (2008). Higher antioxidant activity of lemon juices could possibly be due to pre-concentration of active constituents by application of vacuum freeze drying. Freeze drying of the juice causes dehydration at low temperature that leads to increase in the concentration of valuable components and consequently enhances antioxidant activity (Shofian *et al.,* 2011). Korus (2011) reported the effect of freeze drying in kale leaves (Brassica oleracea L. var. acephala) with increased antioxidant activity. The significant variations in the DPPH scavenging activity for juice was observed. Variations in the antioxidant activity could be because of the diverse and complex nature of antioxidants compounds present in lemon juices that can be described by considering the factors such as cultivar, growing conditions, maturity and environmental temperature (Iqbal and Bhanger, 2006).

Interpretation of the Mid-FTIR spectra

Group FTIR spectra of lemon juice extracts in the range of 4000–600 cm^{-1} are shown in Figure 2. The spectra shows absorption bands corresponding to the functional groups vibrations of different compounds including phenolics and other nutrients and non-nutrients comprising vitamins, minerals, dietary fiber, carotenoids and essential oils, which are the main components of lemon fruits (González-Molina *et al.,* 2010).

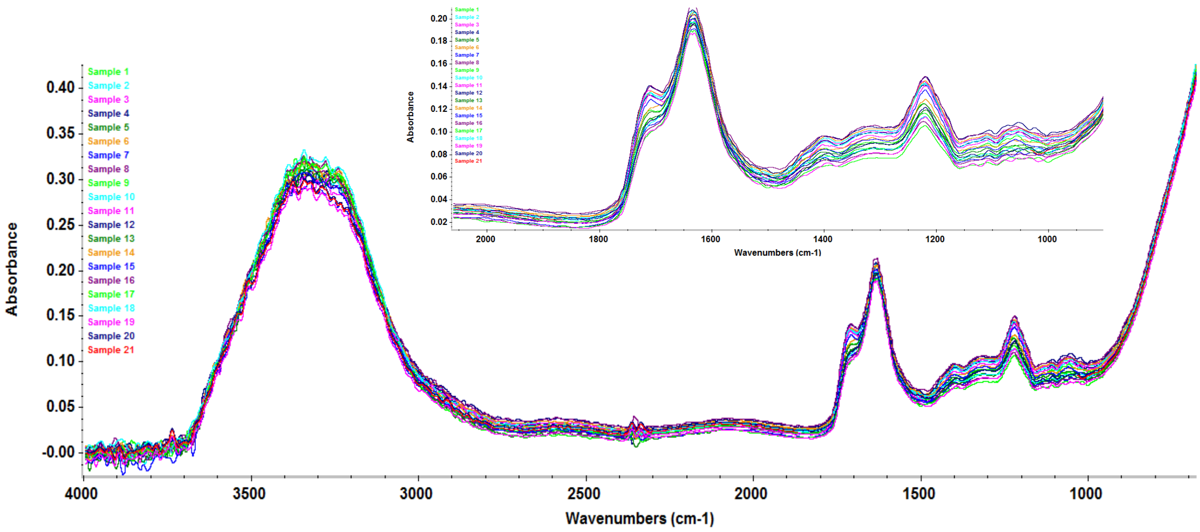

Figure 2: Group FTIR Spectra of lemon juice sample extracts

Many infrared bands originate from the vibrations of the C-H, C-C and C-O bonds in the fingerprint region, which is characteristic for each organic molecule and provides useful information. The polyphenolic bands are generally assigned to this region. The bands ranging from 1130-1040 cm^{-1} and 1270 to 1180 cm^{-1} are characteristic to C-O and OH vibrations of phenolic compounds. The band around 1370 to 1190 cm^{-1} is due to CH_3 symmetrical vibrations and the region ranging from 1500 to 1340 cm^{-1} is assigned to the O-H vibrations (Lam et al. 2005). The carbonyl

(C=O) stretching vibration absorption band appears at 1750–1710 cm^{-1} (Domínguez-Martínez *et al.,* 2014). The bands around 1650 cm^{-1} is assigned to the C = C bond vibrations that are typical of the aromatic compounds. The occurrence and distribution of these functional groups with chemical structures of polyphenols effect the antioxidant properties in fruits (Lam *et al.,* 2005). The fingerprint region of collected FTIR spectra spectra reging between 1900–950 cm^{-1} was found to be optimal by comparing the regression results in terms of correlation coefficient (R) and root-mean-square error of cross-validation, that should be of low value (Leopold *et al.,* 2012). The region from 4000 to 2000 cm^{-1} contains only aliphatic C-H absorption and broad hydroxyl bands, which are of little information, hence were not included for further consideration.

Development of calibration models

For evaluation of antioxidant potential of lemon juice extracts, PLS and PCR algorithms were developed under optimized parameters using TQ Analyst. The best algorithms model was selected on the bases of smaller root mean square error of calibration (RMSEC) and the higher determination coefficient (R^2) that shows the direction and strength of the correlation between predicted and the actual values (Domínguez-Martínez *et al.,* 2014). Errors evaluation of developed models was determined by calculating the residual mean standard error of calibration (RMSEC) by comparing the actual values with the predicted one. The calibration curve with percent difference plot between the actual and predicted values obtained through software for both of the models is shown in Figure 3.

The statistical evaluation of the chemometric models (PLS and PCR algorithms) for predicting the antioxidant activity of lemon juices is shown in Table 1. The R^2 value for the PLS model observed was 0.99864 that was higher than obtained by the PCR model (0.89082). The RMSEC values for PLS and PCR models obtained were 0.171 and 1.53 respectively.

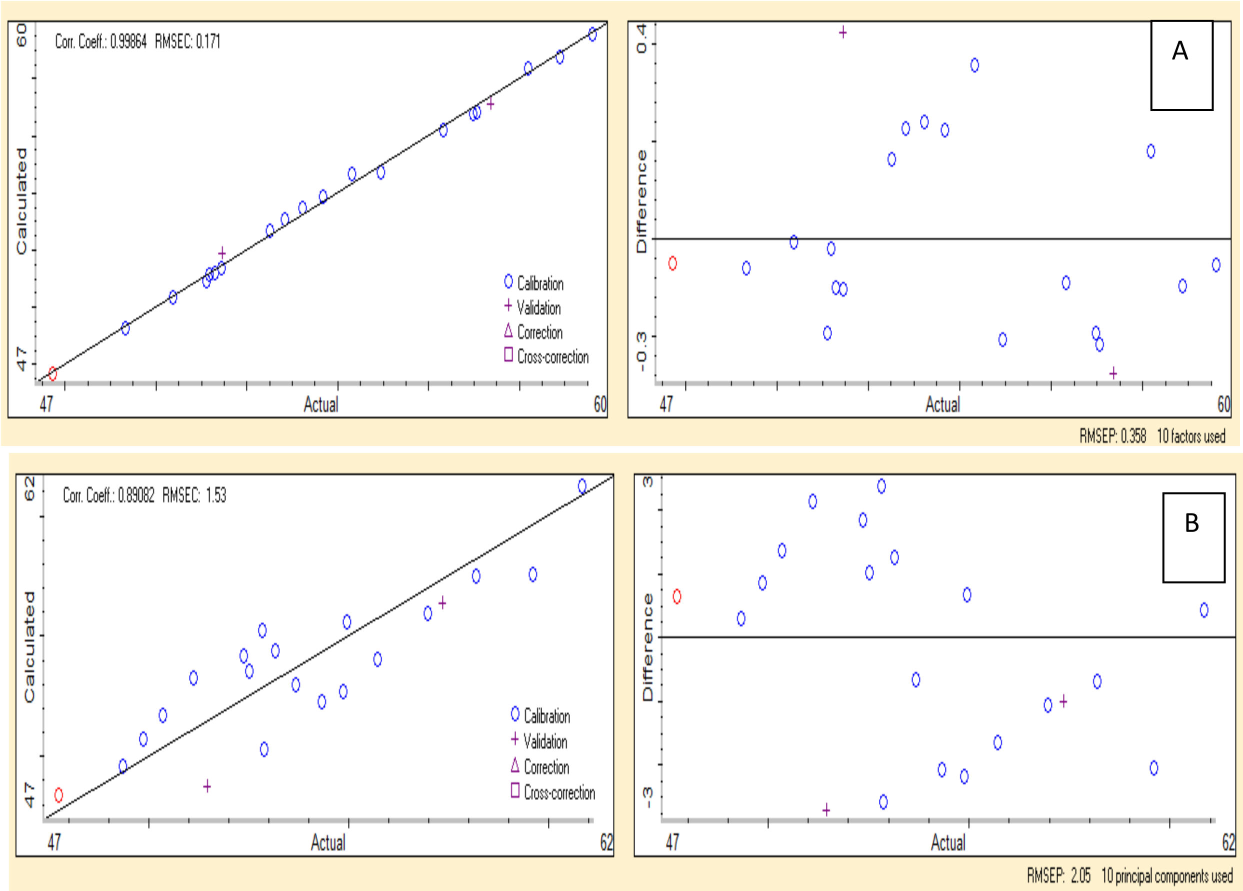

Figure 3: TQ Analyst PLS (A) and PCR (B) algorithms models based on FTIR Spectra

Table 1: Calibration data of the models developed with PLS and PCR algorithms to evaluate antioxidant activity of lemon juice extracts

Algorithm	PLS	PCR
Spectral region (cm^{-1})	1900-950 cm^{-1}	1900-950 cm^{-1}
Number of factors [a]	10	10
RMSEC [b]	0.171	1.53
RMSECV [c]	1.91	3.62
RMSEP [d]	0.358	2.05
R^2 [e]	0.99864	0.89082

[a] Optimal factors (PLS) or optimum principal components (PCR).

[b] Root mean square error of calibration (RMSEC) should be as low as possible.

[c] Root mean square error of cross validation (RMSECV) should be as low as possible.

[d] Root mean square error of prediction (RMSEP) should be as low as possible

[e] Coefficient of determination (R^2) should be as close to 1 as possible.

The PLS model shows comparatively the better calibration strength than the PCR model as it revealed higher R^2 and lower RMSEC values as compared to the PCR model, hence, the PLS algorithm was best calibration model achieved. The number of factors are part of calibration models, which are required to obtain a minimal variation constant. These number of factors are an important parameter of the PLS and PCR algorithms. Normally more factors are required for PCR than PLS, which is one of difference between the two algorithms. In order to achieve a noise free spectrum, the number of factors should be less than half the number of the calibration set samples (Domínguez-Martínez *et al.,* 2014). The results of antioxidant activity based on DPPH standard method and FTIR-PLS developed method are quite comparable with a correlation factor of 0.994 as shown in Figure 4. The low values of the RMSEC and RMSEP of 0.171 and 0.358, respectively, estimate the precision of the antioxidant activity prediction (Leopold *et al.,* 2012).

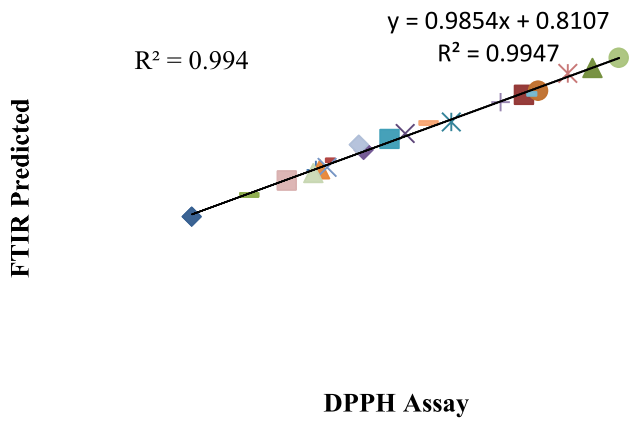

Figure 4: Correlation of antioxidant capacity predicted by Fourier transforms infrared spectroscopy (FTIR) versus DPPH Scavenging assay

CONCLUSION

In this study attempt is made to establish the possibility of using ATR-FTIR technique combined with chemometrics for determining the Antioxidant activity of freeze-dried lemon juice extracts. The good correlation between antioxidant activities predicted by proposed FTIR–PLS methods and determined by the reference DPPH radical scavenging assay, prove the potential of FTIR spectroscopy as a simple, precise and cost-effective tool for evaluation of antioxidant activity of lemon juices. The results show that FTIR spectroscopy in conjunction with chemometrics can be successfully used as an alternative, uncomplicated and fast method for the evaluation of antioxidant activity of different fruits and foods and medicinal plants.

ACKNOWLEDGMENT

The authors gratefully acknowledge the research facilities provided by National Centre of Excellence in Analytical Chemistry, University of Sindh, Jamshoro, Pakistan.

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