Application of Fourier-Transform Infrared (FT-IR) Spectroscopy for Determination of Total Phenolics of Freeze Dried Lemon Juices

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Summary: A cost effective and environmentally safe analytical method for rapid assessment of total phenolic content (TPC) in freeze dried lemon juice samples was developed using transmission Fourier-transform infrared spectroscopy (FT-IR) in conjunction with chemometric techniques. Two types of calibrations i.e. simple Beer's law and partial least square (PLS) were applied to investigate most accurate calibration model based on region from1420 to 1330 cm^{-1}. The better analytical performance was obtained by PLS technique coefficient of determination (R^2), root mean square error of calibration (RMSEC) with the value of 0.999 and 0.00864, respectively. The results of TPC in freeze dried lemon juice samples obtained by transmission FT-IR were compared with TPC observed by Folin-Ciocalteu (FC) assay and found to be comparable. Outcomes of the present study indicate that transmission FT-IR spectroscopic approach could be used as an alternative approach in place of Folin-Ciocalteu (FC) assay which is expensive and time-consuming conventional chemical methods for determination of the total phenolic content of lemon fruits.

Keywords: Fourier-Transform Infrared (FT-IR); Lemon juice, Total phenolic content, Quantitative analysis, Folin-ciocalteu assay.

Introduction

Clinical trials and epidemiological studies reported that a high dietary intake of fruits and vegetables is strongly related with a reduced risk of some chronic diseases, such as various types of cancer, cardiovascular disease, type II diabetes and other degenerative or age-related diseases [1, 2]. It is suggested that this may be due to the presence of antioxidant compounds in fruits that protects the cells against the oxidative damage caused by the reactive oxygen species (ROS) and reactive nitrogen species (RNS). In addition to vitamins (C and E) and carotenoids, the bioactive non-nutrient phytochemicals, such as phenolic compounds, are supposed to play an important role in the prevention of major chronic diseases [2, 3]. Actually, phenolic compounds are major antioxidants that play very important role in the diet [3]. Several types of plant materials, such as vegetables, fruits, seeds, hulls, woods, barks, roots and leaves, spices and herbs, etc. are considered to be potential sources of antioxidants. The antioxidant compounds from natural sources could be used for increasing the stability of foods by preventing lipid peroxidation and protecting oxidative damage in living systems by scavenging oxygen radicals [4].

Lemon (*Citrus limon*) being an important member of citrus fruits which is a rich source of many bioactive components including phenolics [5]. In addition to other soft drinks, lemon juice can also be used as a source of citric acid. It is a legal additive in beverages as an acidifier and preservative in fruit juices and foods [6].

The Folin-Ciocalteu (F-C) assay has been used for a long time as a measure of total phenolics in botanical samples. This method is based on a chemical reduction of a reagent, a mixture of tungsten and molybdenum oxides yielding a bluish compound that is measured at 750 nm [7]. Singleton and Rossi [8] improved the previously developed method that originated from chemical reagents used for tyrosine analysis [9]. The improved method outlined by Singleton and Rossi [8] specified the conditions to minimize variability and eliminate unpredictable results. Nevertheless, very few papers published followed the exact steps of the improved FC method. The FC method suffers from a number of interfering substances particularly sugars, aromatic amines, sulfur dioxide, ascorbic acid and other enediols and reductones, organic acids, and Fe (II), and correction for interfering substances should be made. Additional nonphenolic organic substances and some inorganic substances may also react with the F-C reagent to give elevated apparent phenolic concentrations. Lack of standardization of methods

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can lead to several orders of magnitude difference in detected phenols. Hence, continue efforts to standardize the assay are clearly required [10].

In the last few years, numerous foods have been characterized by infrared spectroscopy and multivariate calibrations have been conducted [11]. These methods present some advantages such as versatility, efficiency and speed, easy sample preparation with reduced or no pre-treatment steps and the prediction of chemical and physical sample parameters from a single spectrum based on the use of multivariate calibration [12]. FTIR spectroscopy has been used for quantification of different food components such as glucose, fructose and organic acids [7], however, for the evaluation of total phenolic content by FTIR in combination with multivariate calibration has not been yet reported. Our group has already developed FTIR methods for determination of various components in many matrixes free fatty acids from poultry feed lipid extracts [13], pharmaceutical formulations [14, 15] and for categorizing the main fatty acid classes in vegetable oils [16]. The aim of present study was to develop a fast, inexpensive, simple and reliable method for rapid screening of TPC in lemon juice samples using infrared spectral data in combination with multivariate calibration, eliminating the use of toxic solvents with the advantage of green chemistry.

Experimental

Samples Preparation

Lemon samples (n = 15) were collected from local market of Hyderabad, Pakistan during 2013-2014. Fruits without visible damage or physiological defects were selected and were cleaned, washed, peeled off and de-seeded. The remaining part was mechanically squeezed to extract the juice. Extracted juice was freeze dried at -40 ^{0}C using Labconco Freeze zone 4.5, until a brownish cohesive and physically stable product was obtained. The freeze-dried samples were kept in an air-tight container and stored at 4 ^{o}C for further analysis.

Chemicals and Reagents

HPLC-grade methanol and ACS-grade HCl were purchased from Fisher Scientific Chemicals (USA) and Folin-Ciocalteu phenol reagent, sodium carbonate and gallic acid were acquired from Sigma-Aldrich. All other chemicals and reagents used were of analytical grade. The solution preparations were carried out using double distilled water.

Extraction of Phytochemicals

Freeze dried juice samples were extracted thrice with 80% (v/v) methanol in an ultrasonic bath for 20 min at ambient temperature. The methanol extracts were filtered and extraction was repeated twice by adding 1ml of 0.15% HCl. All portions of individual extracts were combined, concentrated by removing the solvent and freeze dried. The dried extracts were stored in an air tight container at -20 ^{o}C prior to analysis.

Calibration and determination total phenolic content by Folin-Ciocalteu method

Determination of total phenolic content in the methanol extracts of lemon juice samples was evaluated using a colorimetric method reported by Singleton & Rossi [8] with slight variations. The method involves the formation of a blue complex followed by reduction of Folin-Ciocalteu reagent by samples containing phenolic compounds. Standards of gallic acid were prepared in the range between 0.1 to 1.0 milligrams of gallic acid in 80% methanol. From each prepared gallic acid standard, 0.2 ml was mixed with 1.0 ml of diluted Folin–Ciocalteu reagent and allowed to react for 5 min in the dark. 2.0 ml of saturated sodium carbonate solution was added to each reaction mixture and diluted to 7 ml with deionized water. The final mixture was mixed thoroughly and incubated for 30 min at 37 ºC. The absorbance of each mixture was measured at 747 nm with a Perkin Elmer Lambda-35 UV-spectrophotometer. Appropriately diluted Lemon samples extracts were prepared following the same procedure used for preparation for gallic acid standards. The total phenolic content in the extracts was expressed as milligrams of gallic acid equivalents per gram of weight of sample (mg GAE/ g WS).

Standard and Sample Preparation for FTIR Analysis

Gallic acid is normally used as standard in FC method and results are expressed as equivalents of gallic acid standard relative to dry weight of samples [17, 18]. A range of different concentrations of gallic acid was prepared in methanol (80% v/v) and were scanned on Thermo Nicolet 5700-FTIR spectrometer using CaF_2 transmission cell containing a spacer size of 0.5 mm. Under the same parameters, appropriately diluted Lemon sample extracts were prepared and analyzed for total phenolic content. Results were expressed as mg of gallic acid equivalents (GAE) per gram of sample (mg GAE/g).

FT-IR spectral measurements

Infrared spectra of standards as well as samples were recorded in triplicate using Thermo Nicolet 5700 FTIR spectrometer equipped with deuterated triglycine sulfate (DTGS) detector. The instrument was controlled by commercially available IR spectra analysis software package OMNIC (Thermo Nicolet Analytical Instruments, Madison, WI). All spectra were recorded in the range of 4000–400cm^{-1} averaging 32 scans at a resolution of 4 cm^{-1}. The spectrum of each standard as well as sample was ratioed against a fresh background spectrum recorded from methanol (80%) [14].

FT-IR Calibrations

Using the Omnic software and TQ Analyst program, calibration models based on simple Beer's law and partial least square (PLS) were optimized for the quantitative determination of total phenolic content in lemon samples. In the TQ Analyst program, out of different concentration ranges of gallic acid standard, a range from 0.1 mg to 1.0 mg were selected. This is the same range that was also selected for FC Method. Specific region of phenolic compounds, i.e. 1420–1330cm^{-1} [4] was specified in the TQ Analyst program (Thermo Electron Corporation, Madison, WI, USA, 2004) for best results. For the Beer's law and PLS model, out of ten calibration standards, eight of them were used as a calibration points and two used as a validation points as randomly selected by the software. A very good calibration was generated with excellent regression results. The best calibrations model was chosen based on higher determination coefficient (R^2) that indicate the strength and direction of the linear relationship between predicted and the actual values [12].

Limit of detection and quantification

Limit of detection (LOD), the minimum concentration from which it is likely to assume the presence of the analyte with reasonable statistical certainty and limit of quantification (LOQ) of proposed method was determined by selecting the region measured at low concentrations of standards, until the gallic acid related signal disappeared. The analysis at the lowest amount which produced substantial signal was repeated eight times and evaluated by the following formula [19]:

$$LOD = 3 \times SD \times C / M$$

Where SD is the standard deviation; C is the concentration of analyte and M is the mean band region. While LOQ was determined by the same way by following equation [19]:

$$LOQ = 10 \times SD \times C / M$$

Results and Discussion

Total phenolic content by Folin–Ciocalteu method

The results of the total phenolic content of lemon juice extracts are shown in Table 2. TPC in samples ranged from 6.125 to 12.681 mg GAE/g, with an average value of 9.740 ± 1.835 mg GAE/g of dry weight of extract. Results for all the samples measured show that there are important differences among the samples studied, probably due to difference of agroclimatic conditions, growth periods, postharvest handling, and pre- and postharvest treatments related to the fruits. The cause of variation in the total phenol content cannot be categorically explicated, though as studied before, it is identified that the plant age and maturity are the principal determinants of variation in phenolic content [20]. Furthermore; higher quantity of TPC was observed in all the samples as compared to already published data for TPC in lemon juice [21] indicating the significant effect of freeze drying on the TPC of juice samples. Freeze drying removes the water to the level at which microbial spoilage and deterioration reactions are minimized [22] keeping the heat-sensitive biological compounds and phytochemicals intact, as it is performed at relatively low temperatures [23].

FTIR analysis and selection of spectral region for calibration

For the quantitative estimation of the total phenolics in lemon juice extracts, the wavelength range in which absorbance measurement is to be made must be determined as a first step in the spectral region selection. The spectral range should include characteristic regions, in which the chemical groups related to the species of interest as well as other matrix constituents absorbed. Most ideally, the noise dominated regions should not be included in the analysis. Although this rejection of region may not be entirely possible when absorptions due to analytes overlap, even minor considerations can exhibit in developing a robust model. A proper spectral range can be identified by computing the correlation spectrum for the constituents of interest. Regions that show a high positive correlation should be selected, whereas regions that show low or no correlation should be ignored [12]. Based on this analysis, the regions at 1420–1330cm^{-1} showing the highest correlation between changes in the composition and spectral response, hence, was preferred as working range. Also, in the selected range an overlay of all analysed samples was observed.

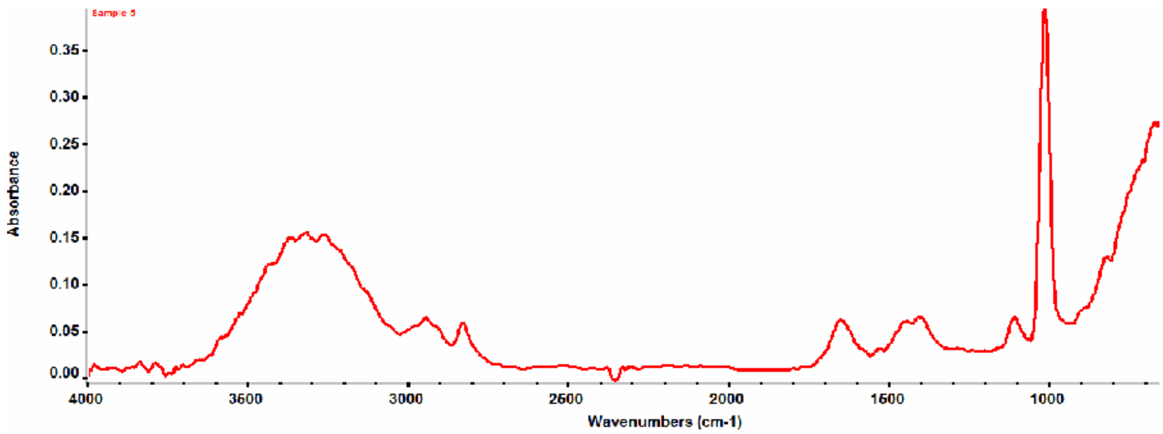

Fig. 1: A representative FTIR spectrum of the freeze dried methanol extract of lemon juice.

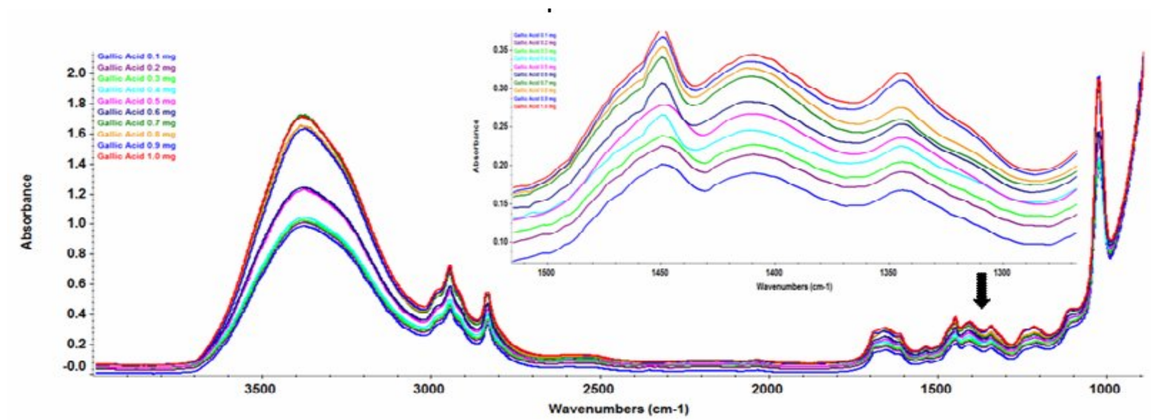

Fig. 2: Group FT-IR transmission spectra of the gallic acid standard.

The intense band detected in the region 3550–2990 cm^{-1} originated from compounds with –OH groups such as water and methanol, in which suitable dilution of samples was carried out (80% methanol), was not useful in this work. Good calibration was generated with excellent regression in the range 1420–1330cm^{-1} that is attributed for O-H inplane deformation absorption band, rather than a band in the range 1650–1450cm^{-1}, that is assigned to phenolic compounds due to the aromatic ring stretching vibration [4]. Since the signal from the phenols can be found in the selected region. The selected spectral region corresponding to the phenyl nuclei (C=C bonds), there are also bands of deformation of $–CH_2–$ groups. The peak at 1376–1373 cm^{-1} is related with symmetric in-plane bending of $–CH_3$. The absorption at 1340–1339 cm^{-1} has been assigned to CH bending and CH_2 wagging. The selection of this region is also supported by the previous studies conducted for prediction of total phenolic content of Moscatel dessert wines [7].

Typical FTIR spectrum for lemon juice sample extract is shown in Fig. 1. Linear response of the absorbance of selected region (1420–1330cm^{-1}) to various concentrations of the gallic acid standard ranging from 0.1mg to 1.0mg by FT-IR transmission spectroscopy in depicted in Fig. 2.

Calibration and quantification of TPC

For evaluation of TPC of lemon juice extracts, simple Beer's law and PLS calibrations were developed using gallic acid standards ranging between 0.10 mg to 1.0 mg using TQ Analyst, program. Excellent linearity (R^2 = 0.99929) was observed in PLS calibration model in comparison to the Beer's law as shown in Fig. 3 and hence PLS model was selected as optimized model for quantification of TPC in lemon samples. Errors evaluation from the calibration was performed by calculating the residual mean standard error of calibration (RMSEC) after comparing the actual concentration with the calculated one for each standard. The calibration curve with % difference plot between the actual and calculated values for the range of calibration obtained through software is shown in Fig. 3.

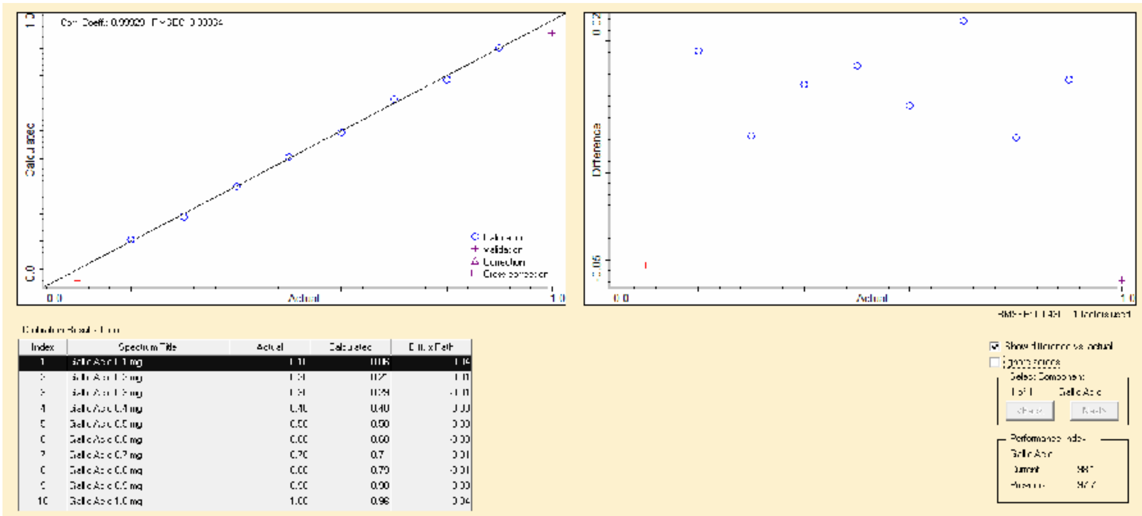

Fig. 3: TQ Analyst calibration of gallic acid standards based on FTIR Spectra and PLS technique.

Table 1 shows the statistics of the developed chemometric models (Beer's law and PLS algorithms). Comparatively, the better model was selected on bases of excellent regression values. The number of factors used in the PLS model was automatically selected by the software and the selection of factor was used to achieve the lowest possible predicted residual error of sum of squares (PRESS) value.

Table-1: Analytical performance of Beer's Law and PLS regression using FTIR method.

	Beers's law	PLS
Spectral region (cm^{-1})	1420–1330cm^{-1}	1420–1330cm^{-1}
Number of factors	-	8
RMSEC	-	0.00864
RMSECV	-	0.0116
R^2	0.99795	0.99929

The minimum sample band that could be determined was three folds as tall as the noise signals and was equal to concentration of 0.03 mg. Therefore, LOD and LOQ determined were 0.03 and 0.07, respectively. These markedly prove the sensitivity of proposed method using transmission FT-IR spectroscopy.

The Infrared spectra of samples recorded under the same parameters of gallic acid standard were opened against the optimized PLS model and each lemon juice extract sample was quantified for their total phenolic content. The results obtained were expressed as mg of gallic acid equivalents (GAE) per gram of sample (mg GAE/g) as shown in Table-2.

The results of TPC achieved by FC standard method and FTIR method are quite comparable on the bases of R^2 value as shown in Fig. 4. These results indicated that FTIR spectroscopy combined with chemometrics can be used for the accurate quantification of TPC in lemon juice extracts.

Table-2: TPC of lemon juice extracts by Folin-Ciocalteau and FTIR method.

Sample	Total Phenolics (mg GAE/g)	
	Folin-Ciocalteu method [a,b]	FTIR method [a,b]
1	10.015 ± 0.09	10.871 ± 0.11
2	7.615 ± 0.23	8.145 ± 0.14
3	9.547 ± 0.21	9.142 ± 0.13
4	12.681 ± 0.14	13.145 ± 0.09
5	8.125 ± 0.16	9.458 ± 0.16
6	9.631 ± 0.18	10.678 ± 0.15
7	10.145 ± 0.08	11.475 ± 0.11
8	6.152 ± 0.11	7.113 ± 0.10
9	8.147 ± .015	8.124 ± 0.11
10	9.145 ± 0.13	9.925 ± 0.09
11	8.785 ± 0.17	8.865 ± 0.09
12	11.754 ±0.21	11.156 ± 0.11
13	10.452 ±0.24	10.854 ±0.16
14	12.128 ± 0.20	12.864 ± 0.13
15	11.780 ± 0.19	12.145 ± 0.14

[a] Values are mean ± standard deviation of sample analyzed individually in triplicate.
[b] (mg GAE/g of dry weight of extract)

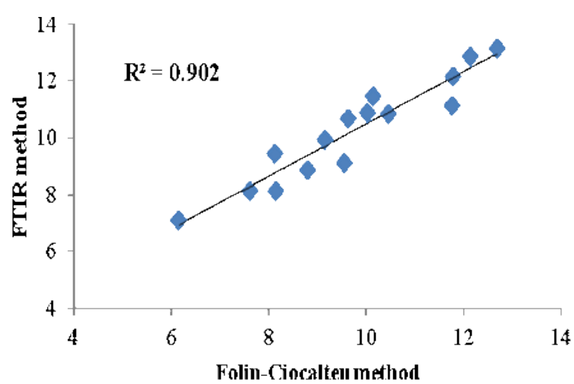

Fig. 4: Comparative results of FC and FTIR methods for TPC determination of freeze dried methanol extract of lemon juices.

Conclusion

Current method for the determination of TPC in lemon juice extracts using FT-IR spectroscopy is a very simple analytical method as it doesn't requires intricate procedures and costly reagents. FTIR spectroscopy in combination with chemometrics has many benefits such as speed, easy sample preparations, environment friendly, cost effective as compared to conventional methods for the determination of TPC in lemon juice extracts. The proposed method is potential choice for the rapid screening of TPC as compare to classical FC method which is laborious and needs a lot of solvents. Furthermore, proposed method could be extended for the determination of TPC in other fruit samples, food ingredients and food products used in food processing industries. Furthermore, current study explored the enhancement of valuable components like phenolics in lemon fruit juices by freeze drying procedure, emphasizing that the freeze dried extract of juices could be utilized in functional foods and food processing industries

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