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Feasibility of using Raman spectroscopy for detection of tannin changes in pomegranate fruits during maturity

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ABSTRACT

Detecting changes in tannin as a major phytochemical content of pomegranate fruit can be used to monitor its maturity. In this study, using Raman spectroscopy as a rapid and noninvasive technique to distinguish four different maturity stages of pomegranate fruits (Immature stage (S1), Fairly Half-ripe stage: (S2), Half-ripe stage (S3) and Full ripe stage (S4)) on the basis of tannin changes was studied. Spectral acquisitions from fruit cross-sections in the wavelength range of 100-3000 cm^{-1} were carried out. A modified polynomial was fitted to the signals to remove the background and obtain desired peaks. Also, Self-modeling Mixture Analysis (SMA(was used to extract Raman signatures of different compositions from corrected spectra. After extracting of the pure component spectra using SMA from all the pomegranate fruit samples, the Spectral Information Divergence (SID) was performed to evaluate the degree of maturity of the pomegranate fruits. It concluded that Raman spectroscopy has a potential of the future development of a Raman-based nondestructive approach for subsurface detection of tannin as an indicator of pomegranate maturity.

1. Introduction

Tannins are a class of astringent, polyphenolic biomolecules that bind to and precipitate proteins and various other organic compounds including amino acids and alkaloids. They are widely distributed in vegetables and fruits (Vargas-Murga et al., 2016). The interest in tannins has considerably increased because of their essential health benefits (Murillo et al., 2013). They are fat-soluble microconstituents localized in organelles called chromoplasts (Brackmann et al., 2011; Schulz, 2016) with important functions, antioxidant properties, and physiological effects, such as to accelerate blood clotting, reduce blood pressure, decrease the serum lipid level, produce liver necrosis, and modulate immunoresponses. Thus, the growing interest in tannins has increased the searching for new natural sources, including many underutilized wild vegetables and fruits which can serve as biological sinks of phytochemicals with nutritional properties and health benefits (Delgado-Pelayo et al., 2016). In this context, many tropical fruits can be considered a reservoir of bioactive substances with a particular interest due to their possible health-promoting characteristics (Murillo et al., 2013).

Pomegranate fruit (Punica granatum L.) is one of the most productive fruits in the in tropical and subtropical regions such as Iran, Afghanistan, India, Mediterranean countries (Morocco, Spain, Turkey, Tunisia and Egypt) and Middle Eastern countries (Khodabakhshian et al., 2016). Based on FAO statistics, Iran ranks 1 st in pomegranate fruit production in the Middle East and North Africa, 2.7 million hectares of orchards are being harvested in Iran with an annual production of 16.5 million tons (Food and Agriculture Organization, 2010). The pomegranate fruits has a high content of tannins, carotenoids, the phenolics, flavonoid glycosides, flavones, flavonols, flavoxanthin in the fruit (Hmid et al., 2017). Moreover, the concentration of these phytochemicals changes with advancing stage of fruit maturity. The astringent taste of pomegranate fruit during the early stages of maturity is closely associated with the level of tannins content that increases with the maturity of pomegranate fruit (Saad et al., 2012). So to ensure the minimum acceptability of the quality to consumers, developing efficient and nondestructive methods to determine optimum harvesting time is needed for the pomegranate fruit.

During last decades, high-performance liquid chromatography (HPLC) and visible spectrophotometry are the usual methods for tannins determination (Khodabakhshian and Emadi, 2016). Although, new and suitable analytical techniques have been investigated. Among them is the Raman spectroscopy, which is an in situ and fast modern analytical technology that provides multiple analysis, chemical and structural molecular information with minimum requirements for sample preparation (Abbas et al., 2012; Boyaci et al., 2015; Prochazka, 2016). Among the main applications of Raman spectroscopy in food science are the identification of the origin and the characterization of vegetable

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oils, sugars and some bioactive compounds such as carotenoids (Chen et al., 2006; McGoverin et al., 2010; Qin et al., 2012). In addition to these advantages, the method allows identifying species, content, and distribution of carotenoids in a biological system (Huo et al., 2011). Generally, raman spectra could offer many slight changes that allow distinguishing a specific spatial conformation of certain phytochemicals. The possibility of in situ and direct analysis in a non-invasive way and with no pre-treatment requirements make this technique a fast and easy-to-handle alternative to characterize the main components of fruits and vegetables (Feltl et al., 2005; Qin et al., 2012).

The identification of tannins in biological systems has been studied through the analysis of the main characteristic Raman bands, characterized by intense Stokes lines between 650 and 1590 cm-1 (Khodabakhshian and Emadi, 2016), for both, condensed tannins and hydrolyzable tannin content, which are simultaneously detected in Raman spectroscopy. Moreover, Raman scattering method reflects the presence of tannins in human skin and is highly reproducible (Hata et al., 2000).

To contribute to the determination of tannins in food samples, the present study aimed to visualize the nature of the main organic components of immature and mature pomegranate fruits in a noninvasive way. For this goal, it is necessary to determine what changes in internal tannin distribution actually occur within the pomegranates during the maturity. A correlation between the spectral results and the conventional extraction method is aimed. Also, chemometric tools such as the Self-modeling Mixture Analysis are applied for classification of samples by maturity stage. Specific objectives were to:

- use the Raman spectroscopy technique to measure the Raman scattering signals from pomegranates at different studied maturity stages;
- visualize the tannin generation patterns that develop inside pomegranates during the maturity by Raman spectroscopy technique.

2. Materials and methods

2.1. Samples

Pomegranates (Ashraf cultivar) were handpicked from commercial orchard in Shahidabad Village, Behshahr County, Mazandaran Province, Iran. During the harvest seasons (August, when it was possible to squeeze juice from fruit arils – October, commercially full mature stage), the 100 sample pomegranate fruits studied correspond to four maturity stages of 25 samples established the based on the subjective evaluation of the skin texture of the fruit, immature stage: hard texture (S1); fairly Half-ripe stage: Fairly firm texture (S2); half-ripe stage: firm texture (S3), and full ripe stage: soft texture (S4) (Fig.1). The harvest of each maturity stage was made at 88, 109, 124 and 143 days after full bloom (DAFB), respectively. Immediately after harvesting the fruits were placed in boxes to protect from injuries. Before spectral acquisition, the required quantities of pomegranates fruits in each maturity stage were cut and allowed to warm with room temperature for approximately 2 h (Mohsenin, 1986). The photo of their cross section is shown in Fig. 2.

As mention before, the Raman spectra of tannin was measured to provide reference spectra. Tannin extract was provided by Silvachimica (Italy).

2.2. Tannin assay

The analyses of tannin content in pomegranates were performed according to the International Pharmacopoeia and AOAC methods [Horwitz and Latimer, 2010] with some modifications. 3 g of sample powder (Rind, aril or spongy white tissues) was infused with 250 mL of deionized double distilled water and then it was filtered through 0.45 μm sample filter. 25 mL of the infusion was added into 1 L conical flask and then 25 mL of indigo solution [0.6%] and 750 mL deionized distilled water was added. The solution has been titrated with 0.1 N aqueous solution of $KMNO_4$ until the blue colored solution changed to golden yellow one. Standard solution of indigo carmine was prepared as following: 6 g indigo carmine was dissolved in 500 mL of deionized distilled water by heating and after cooling 50 mL of 98% H_2SO_4 was added. The solution was diluted to 1 L with deionized distilled water and then it was filtered through 0.2 μm membrane filter. The blank test was carried out by titration of the mixture of 25 mL indigo carmine and 775 mL double distilled water. All samples were analyzed in duplicates. The tannin percent (%) in the samples were calculated as follows:

$$T(\%) = [V - V_0] \times 0.004157 \times 250 \times 100/g \times 25 \quad (1)$$

where V is the volume of 0.1 N aqueous solution of $KMNO_4$ used in the titration of the sample and V0 is the volume of 0.1 N aqueous solution of $KMNO_4$ used in the titration of the blank sample as mL; 0.004157 is the tan- nins equivalent in 1 mL of 0.1 N aqueous solution of $KMNO_4$; g is the mass of the sample taken for the analysis as gram and 250 is the volume of the volumetric flask.

2.3. FT-Raman spectroscopy system

FT-Raman has three main advantages over dispersive Raman systems: (1) reducing the laser-induced fluorescence that a number of samples exhibit; (2) easing the operation as with a Fourier transform infrared (FTIR) spectrometer; and (3) showing a high spectral resolution with a good wavelength accuracy (Yang and Ying, 2011). The cross-sections of cut pomegranate fruits were studied in the region 100-3000 cm^{-1} by FT-Raman spectroscopy with laser excitation at 1064 nm equipped with a Bruker FRA106 Raman module and Opus 5.5 acquisition software. The size of the laser spot on the fruit sample was approximately 100 μm, the laser power on the sample about 100 mW and by acquiring 1000 scans with a spectral resolution of 4 cm^{-1} (Thermo Nicolet NEXUS 870 spectrometer; Thermo Electron Corp, Madison, Wis., and U.S.A). The Raman spectra is shown in Figs. 3 and 4.

2.4. Chemometric analysis

2.4.1. Spectral data analysis

Removing intrinsic background signals is one of the major challenges for the application of Raman spectroscopy in biological materials. These signals are able to produce magnitude stronger than the Raman scattering, so they have to be removed to make easy quantitative analysis of the spectra. A range of methods anchored in instrumental and computational programming approaches has been developed for removing the underlying background signals baseline in Raman spectra, such as polynomial fitting, wavelet transformation, Fourier transformation, and derivatives (Feltl et al., 2005). Recently, polynomial fitting method has been widely used because it is simple, fast and can automatically identify background regions and finally the results are comparable with other methods for background subtraction (Qin et al., 2011, 2012). Modeling spectra using different degrees of polynomial functions is basically carried out in this method. In this study, a modified polynomial (Lieber and Mahadevan-Jansen, 2003) which can fit to the signals and able to remove the background for obtaining desired peaks was investigated. The method is able to omit fluorescence background while keeping Raman features. For each repeat, all data points are allocated to the original values if their intensity values are higher than those of the corresponding points in the original spectrum. The outcome of each trial is a curve which its polynomial coefficients can be obtained by a least squares method. This route will continue until approaching a union for all data points in a trial. Moreover, 1-normalization was performed on the spectra, resulting in a uniform quantitative procedure. Savitzky-Golay 2nd order polynomial and moving average (segment size of 5) algorithms carried out to take the first derivative and smooth the signals. Due to the narrow vibration

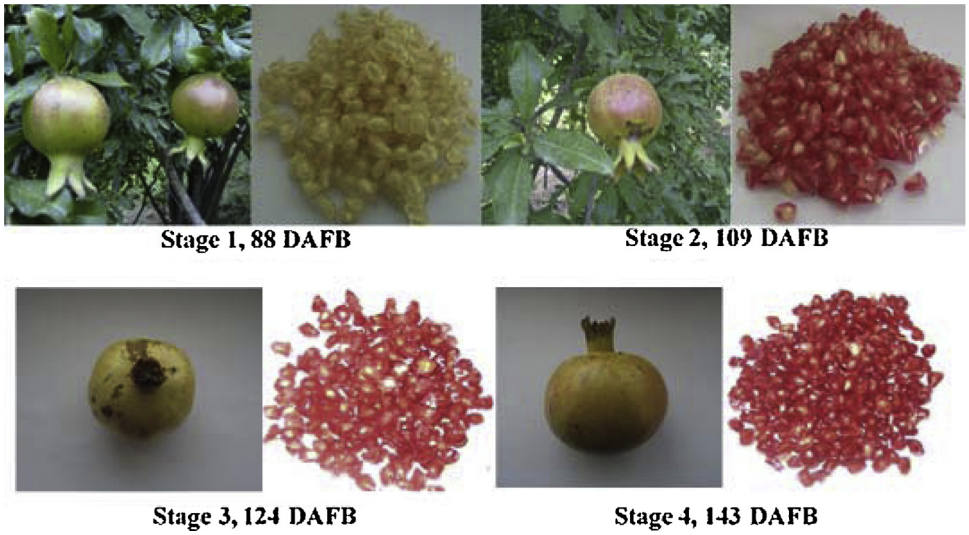

Fig. 1. Fruit and arils of pomegranate (Ashraf cultivar) at different maturity stages. Immature stage: 88 DAFB; half-ripe stage: 109 DAFB; fairly half-ripe stage 124: DAFB; and full-ripe stage: 143 DAFB.

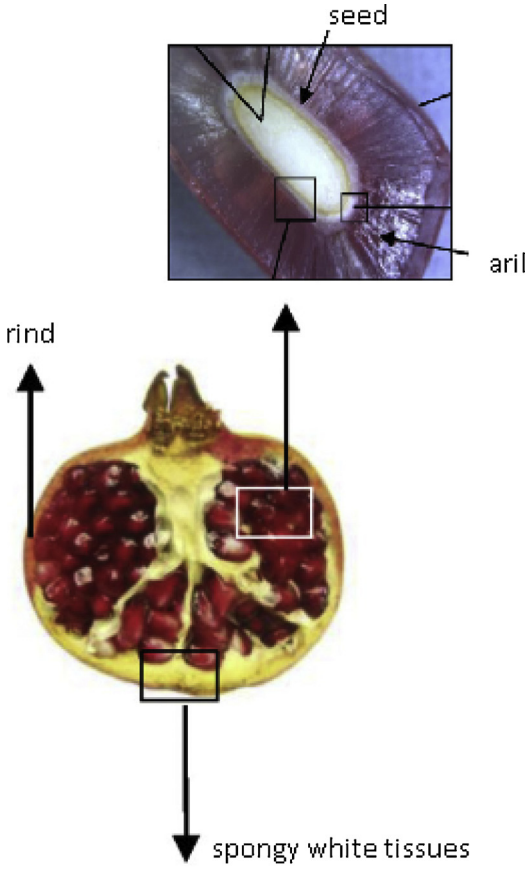

Fig. 2. Identifications of the pomegranates fruit parts at stage 4.

bands assigned to specific molecular vibrations, one must expect spectral overlap between chemical components. Deleting fluorescence background by applying the mentioned curve-fitting method of eight-order polynomial to all Raman spectra was carried out in next step. The procedures described above were executed using programs developed in MATLAB (MathWorks Inc., Natick, MA, USA).

The Raman spectra of studied samples included mixed composition chemical information. Self-modeling Mixture Analysis (SMA(was used to extract Raman signatures of different compositions from corrected spectra. SMA can identify the involvement of each component spectra by disintegrating a data matrix. Also there is no need to know the prior spectral information of each component to resolve a mixture of compounds using SMA. Purity function in PLS-TOOLBOX was used to perform SMA for each set of the corrected Raman spectra. The pure spectra were examined to determine optimal numbers of the pure components. After extracting of the pure component spectra from all the pomegranate fruit samples using SMA, the Spectral Information Divergence (SID) was performed to evaluate the degree of maturity of the pomegranate fruits. SID is actually a measuring tool for spectral similarity. It is able to quantify existing divergence between two spectra using the relative entropy to account for the spectral information. The details of necessary algorithms are given by Chang (2000). The Raman spectrum of pure tannin was used as a reference for spectra matching for the nondestructive test. Any relation between the degree of maturity and distribution of SID values was investigated.

2.4.2. Calibration and validation

The 100 pomegranate fruits of studied variety were randomly divided in two categories: the first category including 70 samples was used to develop calibration models and the other was used for predicting tannin and validation purposes. The calibration equations were obtained using partial least squares (PLS) regression method. PLS is a multivariate method, which is widely employed in spectroscopy analysis (Khodabakhshian et al., 2017). Since there is a large amount of spectral information generated by Raman spectroscopy, the reduction of original data dimensions is required to provide a few uncorrelated variables containing only relevant information from the samples. So, before modeling by PLS regression the method of principle component analysis (PCA), a best known and most widely used data reduction method, was employed. PLS analysis can be carried out to create the regression model leading to the content prediction of chemical components. As a result, the PLS method was used to consider simultaneously the variable matrix Y (the tannin percent) and the variable matrix X (the spectral data) for developing calibration models. The internal validation (in groups of 30 samples) was used to validate the models (Khodabakhshian et al., 2017). The accuracy of the calibration and validation were assessed by correlation coefficient (r), root mean square error of calibration (RMSEC), root mean square error of prediction (RMSEP) and ratio performance deviation (RPD) as follows (Khodabakhshian et al., 2017):

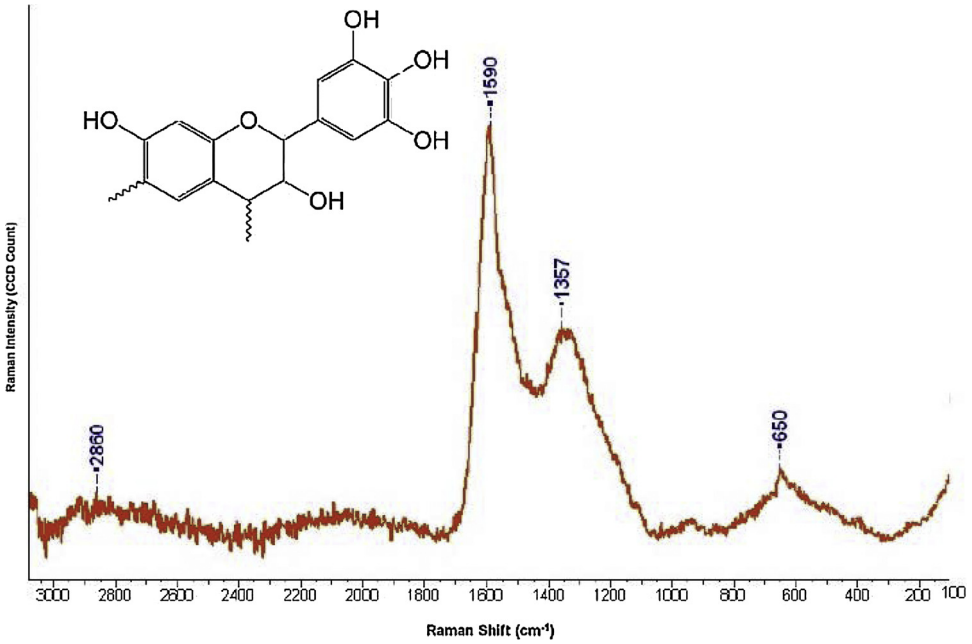

Fig. 3. Reference Raman spectra of tannin.

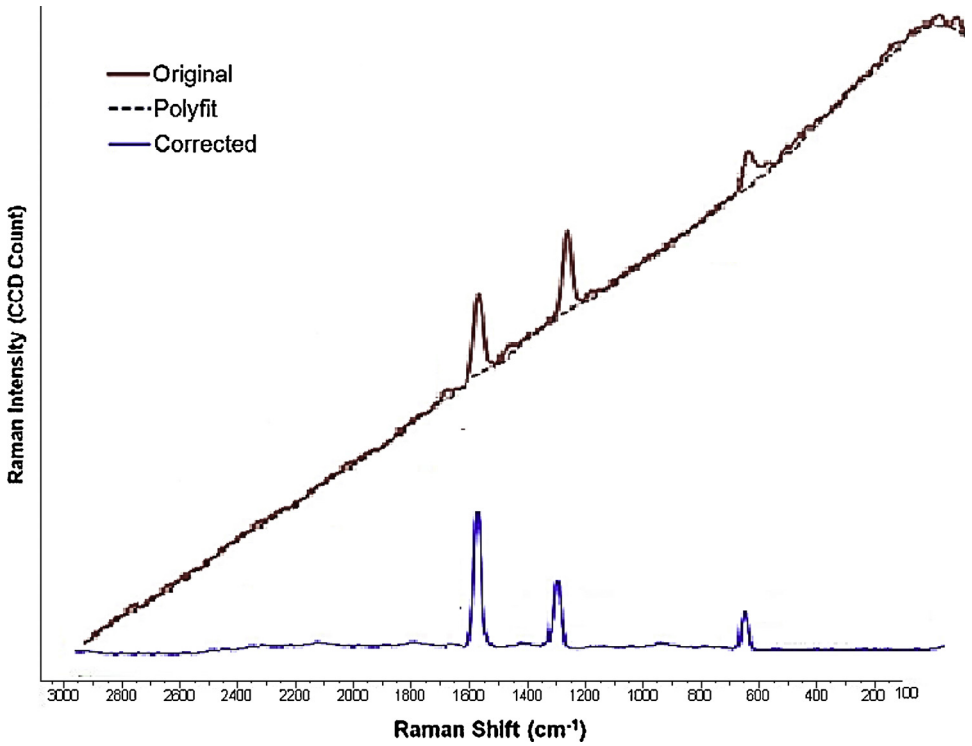

Fig. 4. A typical example of background correction for Raman spectra of pomegrante fruit.

$$r = \sqrt{\sum_{i=1}^{n} (\hat{y}_i - y_i)^2} / \sqrt{\sum_{i=1}^{n} (\hat{y}_i - y_m)^2} \quad (2)$$

$$RMSEC = \sqrt{\frac{1}{n_c} \sum_{i=1}^{n_c} (\hat{y}_i - y_i)^2} \quad (3)$$

$$RMSEP = \sqrt{\frac{1}{n_p} \sum_{i=1}^{n_p} (\hat{y}_i - y_i)^2} \quad (4)$$

$$RPD = \frac{SD}{RMSEC\,(P)} \quad (5)$$

Where ŷi is the predicted value of the i-th observation, yi is the measured value of the i-th observation, ym is the mean value of the calibration or prediction set, n, nc, and np are the number of observations in the data set, calibration and prediction set, respectively. Generally, a good model should have higher correlation coefficients; lower both RMSEC and RMSEP values, but also a small difference between RMSEC and RMSEP or a RPD value should be more than 5(Liu et al., 2010).

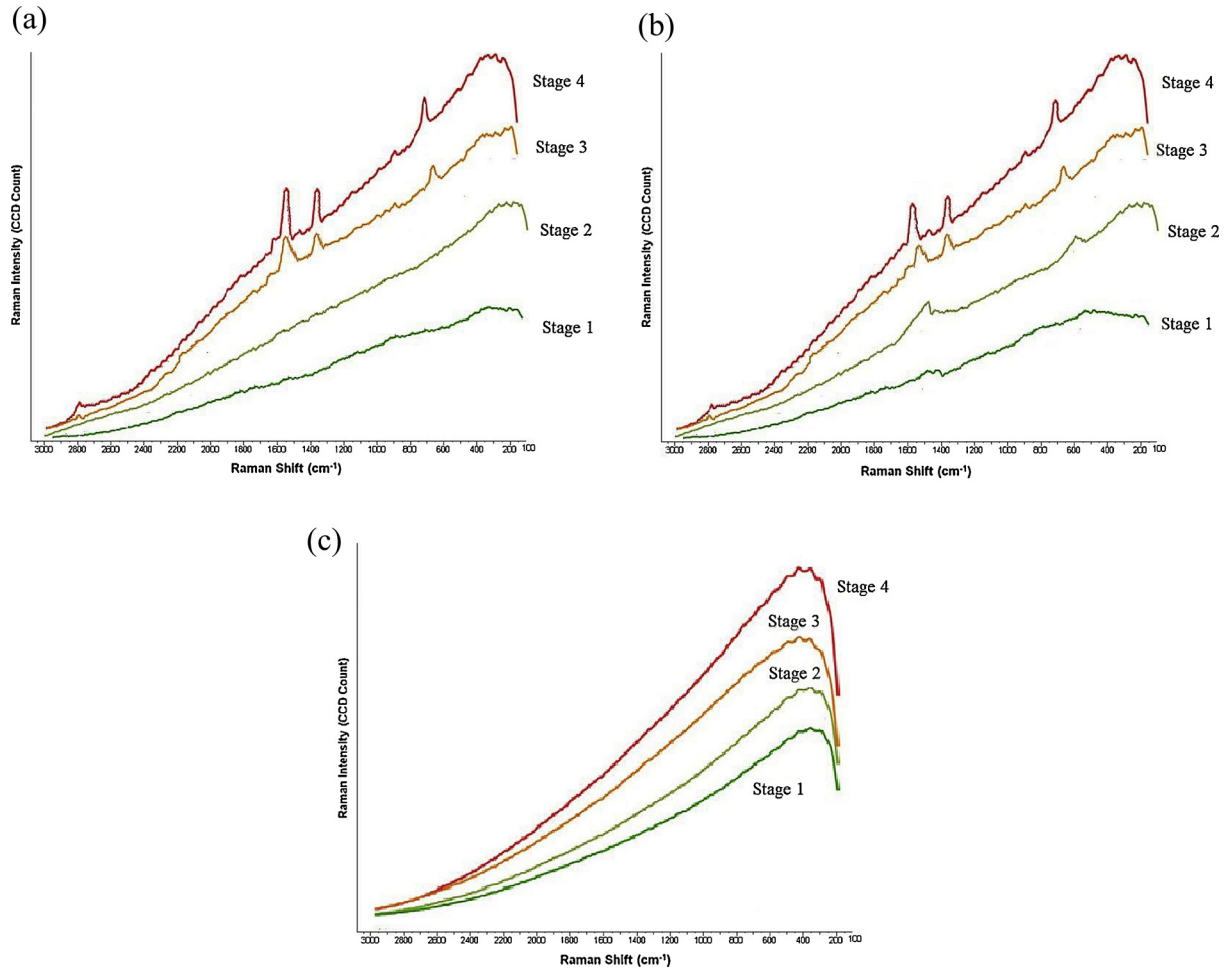

Fig. 5. Typical Raman spectra of major pomegranate fruit parts at selected maturity stages. (Identifications of the fruit parts are illustrated Fig. 2).

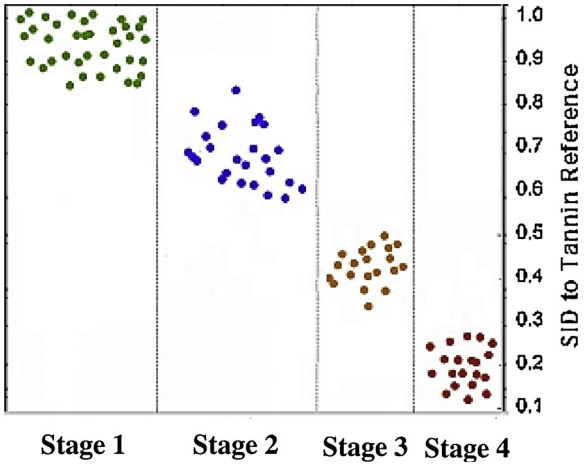

Fig. 6. Spectral information divergence (SID) values between the reference Raman spectrum of tannin and pure component spectra of the pomegranate fruit samples at four studied maturity stages.

3. Results and discussion

3.1. Raman spectra of tannin

Raman features of the tannin in the wavelength range of 100-3000 cm^{-1} are shown in Fig. 3. As shown in the figure, major Raman peaks of the tannin generally occupied two distinct spectral regions. Four Raman peaks of the tannin were observed in the spectral region of 200–1600 cm^{-1}. Only one small peak (around 2850 cm^{-1}) was located beyond 1600 cm^{-1}. The tannin showed its highest Raman intensity at 1590 cm^{-1}, which was higher than that at 1357 cm^{-1}. The other peak (650 cm^{-1}) showed low intensity. As stated by many researchers (Shahidi and Naczk, 2004; Al-Farsi et al., 2005; Biglari et al., 2008), the three bands visible at 1590, 1357 and 650 cm^{-1} can be safely assigned to stretching C═C, C−O−C, and C−H bonds which compose the structure of phytochemicals. The peaks at around 1590 cm^{-1} are related to the C═C stretching within the aromatic rings, while the peaks at around 1357 cm^{-1} are related to the C═C and C−O−C stretching (Tondi and Pizzi, 2009). Also, 650 cm^{-1} is out-of-plane C−H bending and ring deformation vibrations (Tondi and Pizzi, 2009). As it was stated earlier, beyond 1600 cm^{-1}, no notable Raman scattering signals were observed for tannin. Since the main Raman features of the tannin are in the spectral region of 200–1600 cm^{-1}, this region was used in calculating spectral information divergence to evaluate the maturity degree of the pomegranate fruits.

3.2. Raman measurements of pomegranate fruits

The polynomial fitting method was applied to all Raman spectra to remove background signals. A typical example of original and correcting the Raman spectra of samples at three wavelengths corresponding to tannin peaks are shown in Fig. 4. The performance of the modified polynomial curve-fitting method is proved by using these spectra. As found in the figure, the fitting method produced a good fit for the background signals at both high and low intensity levels.

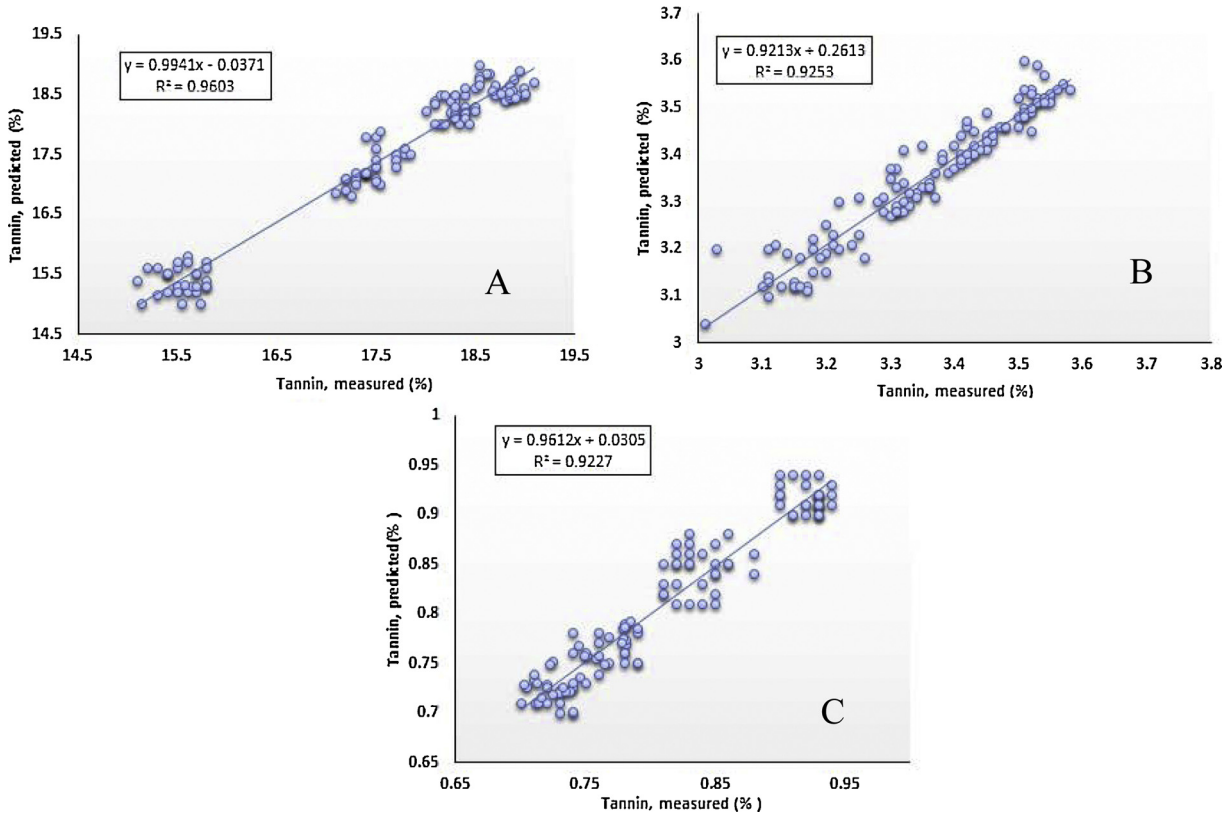

Fig. 7. Scatter plot of measured values versus Raman predicted values for the validation set after application of preprocessing techniques: A: Rind; B: Aril; C: and Spongy white tissues.

Removal of the signal baseline improved the Raman peaks as can be seen in the corrected spectra.

The representative Raman spectra measured for areas of the aril, rind and spongy white tissues parts of pomegranates at four studied maturity stages are shown in Fig. 5. As it was seen from this figure, all the spectra shared in common a broad peak around 300 cm^{-1}, the result of autofluorescence from the laser–pomegranate interaction. This background signal showed the highest intensity for Spongy white tissues spectra and the lowest intensity for rind spectra. Among spectra for the same sample part, the background intensities varied randomly. Three Raman peaks were known in the corrected spectra, happening at the same wavelength as those for peaks of pure tannin (ie., 650, 1357 and 1590 cm^{-1}), was clearly observed in the spectra for aril and rind (Fig. 5a and b) at the more mature stages (stages 3 and 4). Neither aril nor rind spectra show Raman peaks of tannin for samples at stages 1 and 2, suggesting no tannin presence in the fruit at these stages. Because of the increasing tannin content in the more mature fruit samples, the intensities of the Raman peaks generally increased in the aril and rind as the pomegranate matured. Owing to the lack of tannin, the spongy white tissues spectra did not show peaks at any stage of four studied maturity stages (Fig. 5c).

The most bands explored in the region of 650 to 1357 cm^{-1} of the pomegranate fruit can be attributed to existing chlorophylls, hydrolysable polysaccharides and waxes in the cuticular membrane of this fruit (Shahidi and Naczk, 2004; Hmid et al., 2017). These polysaccharides, which are associated with the plant cell wall and are responsible for the elastic modulus, stiffness and the linear elastic behavior of the cuticle, are pectins, hemicelluloses and celluloses. Raman peaks around 650 cm^{-1} is closed to absorption bands of −CH and −OH functional groups which can be due to absorption by water and carbohydrates in pomegranate (Hmid et al., 2017). Another absorption around 1357 cm^{-1} is related to −CH 2nd overtone and finally absorption around 1590 cm^{-1} can be related to absorption of −CH and −OH functional groups. Overall, the corrected spectra with a flat baseline followed patterns of the refer-spectrum of pure tannin. Then identifying tannin changes in pomegranate fruits by spectral information divergence can be applicable. Combination of these results with findings from sample-destructive measurements increased the possibility of non-destructive detecting of maturity of pomegrante fruit by a Raman-based method.

3.3. Evaluation of the maturity stage in pomegranate fruits on the basis of tannin changes

The changes of Raman scattering intensity during the maturity process supply a possible means of evaluating the degree of maturity of pomegranate fruits. As tannin content increases with increasing pomegranate fruit maturity to highest level in stage 4, the degree of pomegranate fruit maturities can be assessed by calculating the spectral differences between pomegranate fruit and the pure tannin. Fig. 6 represent the spectral information divergence values between the pure component spectra of all the studied samples and the reference Raman spectrum of the tannin. SID values are influenced by absolute position and the relative intensity of the Raman peaks in the component spectra. As found from this figure, the SID values steadily increase from the stage 1 to the stage, 4 demonstrating the rising trend of the spectral differences with respect to the pure tannin. The fruits at stage 1 had high SID values due to their lack of Raman peaks from tannin. Due to the appearance of tannin in pomegranates fruits at stage 4, the highest level make reaching of SID values to their lowest overall level at this stage.

3.4. Validation of Raman spectroscopy results and its comparison to destructive analysis

Maximum values of r and minimum values of RMSEC and RMSEP

values and also a high RPD value (best model) for the prediction of tannin in PLS regression was developed when SNV, median filter and first derivative were used as preprocessing (Fig. 7). Raman spectroscopy in conjunction with PLSR could broadly predict the amount of tannin per pomegranate sample. This is the first reported prediction of tannin in the literatures, to the best of our knowledge, with the best model displaying correlation coefficient and a RMSEP.

4. Conclusion

This study reports the potential of FT Raman spectroscopy for nondestructive discriminating of pomegranate fruits according to the four maturity stages. The analysis of the Raman signal changes that happening during pomegranate maturity and its relationship with the maturity degree of the pomegranate fruits was studied. In this regard, changes of pure tannin content in the wavelength range of 100–3000 cm^{-1} as a good maturity index for pomegranate fruits was investigated. A modified polynomial, Self-Modeling mixture Analysis (SMA(and the Spectral Information Divergence (SID) was performed on different samples at four maturity stages. The following conclusions can be drawn from our results:

1 Raman spectra of samples at four maturity stages illustrated the patterns same as Raman spectra of pure tannin during the maturity process. Three Raman peaks were known in the corrected spectra, happening at the same wavelength as those for peaks of pure tannin (650, 1357 and 1590 cm^{-1}).
2 The position and intensity changes of the Raman peaks can be quantified by the spectral information divergence method using pure tannin as the reference. The SID values decrease from the stage 1 to stage 4. It can be used to evaluate the maturity degree of the pomegranate fruits.

The results obtained in this study demonstrate that Raman spectroscopy as the chemometric tool has an acceptable potential to evaluate maturity in pomegranate fruit. However, further studies are needed to evaluate this method.

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