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# Rapid detection of green pea adulteration in ground pistachio nuts using near and mid-infrared spectroscopy

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## Abstract

Pistachios nuts can be a target of economically motivated adulterations due to its high economic value. Although these adulterations can be determined by traditional methods, alternative methods such as spectroscopic techniques have been under investigation. For this purpose, Near-infrared (NIR) diffuse reflectance and Mid-infrared-attenuated total reflectance (MIR-ATR) spectroscopy were evaluated to determine the dried green pea seed adulteration in ground pistachio nuts due to their advantages. IR spectroscopy is known as a simple, rapid, non-destructive, high throughput, and environmentally friendly analytical technique. In this study, sixty-three samples (51 for calibration and 12 for external validation sets) of ground pistachio nuts were deliberately adulterated with varying levels of dried green pea seeds (ranging from 0 to 50% (w/w)). Subsequently, both NIR and MIR-ATR spectra of the samples were collected separately. The quantitative predictions of green pea ratio in the samples were achieved using PLSR (Partial Least Squares Regression). Based on the PLSR models, SEP (Standard error of prediction) values of the models were 2.55 and 9.14% for NIR and MIR-ATR, respectively. The rPred (Correlation coefficient of prediction) values of the models were 0.99 and 0.80 for NIR and MIR-ATR, respectively. Additionally, the nondimensional values of RPD (Residual Predictive Deviation) for NIR and MIR-ATR spectra were calculated as 5.7 and 1.6, respectively. These results showed that the NIR-based models provided a distinct advantage over MIR-ATR-based models in accurately estimating the ratio of the dried green pea seeds in binary mixtures. Therefore, NIR spectroscopy has the potential and could be implemented in the routine applications of green pea detection in ground pistachio nuts.

**Key words:** Adulteration, chemometrics, green pea, mid-infrared, near-infrared, pistachio

## Introduction

Pistachio nuts (Pistacia vera L.) are not only tasty but also nutritious and healthy nuts. Pistachios are a good source of protein, heart-healthy fatty acids, dietary fiber, several minerals (potassium, magnesium, copper, and iron) and vitamins (vitamin K, γ-tocopherol, B6 and thiamin) as well as some phytochemicals including phytosterols, phenolic acids and xanthophyll carotenoids providing high antioxidant and anti-inflammatory potential. Specific reviews by Dreher (2012) and Bulló et al. (2015) were dedicated to the detailed evaluation of the composition, nutrition attributes, and health effects of pistachio nuts. Pistachio nuts have been consumed since prehistoric times, and they are currently used as a functional ingredient in various areas including confectionery, pastry, chocolate, and ice cream products, aside from being consumed as a snack food either fresh or roasted and salted (Çağlar et al., 2017). Unfortunately, as an expensive commodity among the other edible nuts commercially available, pistachios are vulnerable to economically motivated adulteration, particularly when used as a functional ingredient in ground form. Pistachios are commonly adulterated by green pea since the color of green pea kernels exhibits great color similarities to ground pistachios. This similarity makes it difficult to determine the authenticity of the products by traditional methods (Eksi-Kocak, 2016).

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Therefore, adulterated pistachios with less cost and higher profit margin may attract some food producers, manufacturers, processors, distributors, or retailers as an ingredient to be included in the abovementioned products.

Common gold standards employed in the detection of food frauds include analytical methods such as liquid chromatography (HPLC) (Cautela et al., 2008), enzyme-linked immunosorbent assays (ELISA) (Asensio et al., 2008), gas chromatography-mass spectrometry (GC-MS) (Ruiz-Matute et al., 2007), isotope ratio mass chromatography (Kropf et al., 2010; Zhao et al., 2014), and liquid chromatography-tandem mass spectroscopy (LC-MS/MS) (Macmahon et al., 2012). Furthermore, polymerase chain reaction-capillary electrophoresis (PCR-CE) analysis based on a barcode DNA genotyping approach was used to determine green pea adulteration in pistachio nuts (Sen et al. 2020). Although the results of these methods are highly sensitive and accurate, they are time-consuming, costly, and require highly skilled labors and comprehensive laboratories. As an alternative or complementary to traditional methods, infrared (IR) spectroscopy has been successfully employed for food fraud investigations in a great variety of foods in recent years with some of its applications reviewed by Rodriguez-Saona et al. (2016) and Valand et al. (2020).

In the literature, there are limited studies available regarding the detection of green pea adulteration in pistachio nuts. In one of these studies, Raman hyperspectral imaging was assessed as an analytical technique, and quantitative models were developed with strong prediction capabilities, yielding a high coefficient of determinations (R2=0.99) and low root-mean-square error of prediction (0.048%) (Eksi-Kocak et al., 2016). In another study, an untargeted liquid chromatographic method coupled with multivariate analysis was evaluated, reporting successful detection of green pea levels above 2.5% (Çavuş et al., 2018). Additionally, laser induces breakdown spectroscopy (LIBS) was successfully used to determine green pea and spinach adulterations in pistachio nuts (Sezer et al. 2019).

To the best of our knowledge, IR spectroscopy has not been investigated as an alternative technique in detecting adulteration in pistachios. Accordingly, our goal was to develop both NIR- and MIR-based prediction models to quantify the % level of the dried green pea seeds added into ground pistachio nuts and compare their performances and demonstrate their potential alternative or complementary use to traditional analytical methods.

## Materials and Methods

### Materials and Sample Preparation

Roasted pistachio nuts (*Pistacia vera* L.) and fresh green pea (*Pisum sativum* L.) samples were purchased from a local market in Canakkale, Turkey. The pistachio nuts were firstly unshelled and then ground by using a coffee grinder (KG 49, Delonghi, Australia). Since roasted pistachio nuts are low in moisture content (Küçüköner and Yurt, 2003), nuts were ground after unshelling with no further drying. However, the green pea samples were dried in a drying oven (UN55, Memmert, Germany) at 70°C for 18 hours to equilibrate moisture content and then were ground by the coffee grinder (KG 49, Delonghi, Australia).

As shown in Table 1, binary mixtures of ground pistachio nuts and dried green pea seeds (63 samples in total) were prepared as 10 g in total for each sample with the % ratio of dried green pea seeds ranging from 0 to 50% by weight.

**Table 1.** Composition of the binary mixtures of ground pistachio nuts and the adulterating dried green pea seeds

| Calibration Set | | | | Validation Set | | | |
|---|---|---|---|---|---|---|---|
| **Sample Code** | **Amount of Ground Pistachio Nuts (g)** | **Amount of Dried Green Pea Seeds (g)** | **Adulteration Level (%)** | **Sample Code** | **Amount of Ground Pistachio Nuts (g)** | **Amount of Dried Green Pea Seeds (g)** | **Adulteration Level (%)** |
| 1 | 10 | 0 | 0 | 1 | 9.7 | 0.3 | 3 |
| 2 | 9.9 | 0.1 | 1 | 2 | 9.3 | 0.7 | 7 |
| 3 | 9.8 | 0.2 | 2 | 3 | 8.9 | 1.1 | 11 |
| 4 | 9.7 | 0.3 | 3 | 4 | 8.5 | 1.5 | 15 |
| . | | | | . | . | . | |
| . | | | | . | . | . | |
| . | | | | . | . | . | |
| 48 | 5.3 | 4.7 | 47 | 9 | 6.5 | 3.5 | 35 |
| 49 | 5.2 | 4.8 | 48 | 10 | 6.1 | 3.9 | 39 |
| 50 | 5.1 | 4.9 | 49 | 11 | 5.7 | 4.3 | 43 |
| 51 | 5 | 5 | 50 | 12 | 5.3 | 4.7 | 47 |

### Infrared Measurements

Nicolet iS50 Flex Gold infrared spectrometer (Thermo Fisher Scientific, Madison, WI, USA), which contained both FT-NIR diffuse reflectance and FT-MIR-ATR was used to collect the IR spectra of the samples. The Ge-coated KBr (potassium bromide) beam splitter on the device dynamically adjusts the wavelength and allows collecting spectra in both NIR and MIR regions (11000-375 cm$^{-1}$).

### Collection of NIR Spectra

NIR spectra of the samples were collected in reflectance mode using the Nicolet IS50 device equipped with a NIR module containing an InGaAs detector (Indium Gallium Arsenite). A rotating apparatus of the NIR module was used during measurement to collect data that represents the entire sample in the container. About 5 g sample was placed on the glass petri dish. For each measurement, 64 spectra were collected in the range of 10000-4000 cm$^{-1}$ wavelength using a 4 cm$^{-1}$ spectral resolution. The background spectrum of the environment was collected using the empty glass container before each sample. The spectra collection for each sample was duplicated and averaged to obtain the final spectrum. All spectra collected were recorded on a computer by using Omnic 9 software (Thermo Fisher Scientific, Madison, WI, USA).

### Collection of MIR-ATR Spectra

The spectra collection of MIR-ATR was carried out by using the Nicolet IS50 device equipped with DLaTGS/KBr (deuterated L-alanine doped triglycine sulfate) detector and single bounce Zn-Se crystal ATR (attenuated total reflectance) (GoldenGate$^{TM}$, Specac Ltd., UK). The sample was directly placed on the crystal covering the 1.5 mm diameter crystal. Then the pressure was applied by using the apparatus of the MIR-ATR module to establish good and homogeneous contact between the sample and the crystal. For each measurement, 64 spectra were collected in the range of 4000-700 cm$^{-1}$ wavelength using a 4 cm$^{-1}$ spectral resolution. The background spectrum of the environment was collected before each sample. The spectra collection for each sample was duplicated and averaged to obtain the final spectrum. All spectra were recorded on a computer by using Omnic 9 software (Thermo Fisher Scientific, Madison, WI, USA).

### Multivariate Analysis

Due to the complexity of the spectra (particularly NIR spectra due to overlapping overtones and combination bands), the chemometric analysis of collected IR spectra was performed by using Pirouette 4.5 multivariate statistical software (Infometrix, Inc. Bothell, WA, USA). Quantitative prediction models to determine the % level of dried green pea seeds were developed using the PLSR technique, which contains properties of both PCA (principal component analysis) and MLR (multiple linear regression).

A DUPLEX algorithm was employed to split the total number of samples (n=62) into a calibration set (n=51) and an external validation set (n=12). Both internal validation (cross-validation using leave-1-out approach) and external validation approaches were used to validate the calibration models. Subsequently, SEC (Standard error of correlation), SECV (Standard error of cross-validation), SEP (Standard error of prediction), rCal (Correlation coefficient of calibration), rCV (Coefficient of cross-validation) and rPred (Correlation coefficient of prediction) values were used to assess the performance of PLSR models. Furthermore, the RPD ("Residual Predictive Deviation" or also known as "Ratio of Prediction to Deviation") value, which is the ratio of standard deviation (SD) of the reference data in the validation set to the SEP, was calculated. RPD values below 2.3 demonstrate "very insufficient" models and predictions that would not be recommended to be used. RPD values in the range of 2.3 and 3 could be employed for rough screening. RPD values between 3.1 and 4.9 demonstrate that the model can be used for screening. Higher RPD values in the range of 5.0 and 6.4 are taken into consideration as "good" and can be used for quality control application. Finally, RPD values 6.5-8 and above 8.1 are considered "very good" and "excellent" models, respectively, as reported by Williams (2001).

## Results and Discussion

### Spectral Information

#### *NIR Spectra*

The NIR spectra (10000-4000 cm$^{-1}$) of pure pistachio nuts and green pea samples are presented in Fig 1.a with absorption bands highlighted. To better observe the resolving vibrations, the spectra were second derivative transformed (Savitzky-Golay second-order polynomial filter with a 25-point window). The peak identification was made based on the literature (Rodriguez-Saona et al., 2017). The peaks at 4261, 5671, 5825, and 8226 cm$^{-1}$ were sourced from combination bands of C–H and C–O stretching vibrations, the first overtone of C–H stretching and the second overtone of C–H stretching in fats, respectively. This spectral difference between pistachio nuts and green pea could be caused by the variety in the fatty acid content of them since pistachio nuts have higher lipid content than green pea (Küçüköner & Yurt, 2003; Chavan et al., 1999). The peak around 4335 cm$^{-1}$ was due to cellulose and starch bands in carbohydrates. The difference in this peak might be related to the high starch content of green pea compared to pistachio nuts (Ratnayake et al., 2002; Tiwari et al., 2013).

#### *MIR-ATR Spectra*

The MIR-ATR (4000-650 cm$^{-1}$) spectra of pure ground pistachio nuts and dried green pea seeds are shown in Fig.1.b with absorption bands highlighted. Peak assignments were done based on Rodriguez-Saona et al. (2017). Absorption at 997 cm$^{-1}$ was sourced from glycosidic linkage in carbohydrates. This difference between pistachio nuts and green pea could be caused by higher starch and carbohydrates content of green pea (Chavan et al., 1999). The peak at 1045 cm$^{-1}$ was due to starch retrogradation, increased crystallinity in carbohydrates. The band 1045 cm$^{-1}$ is very sensitive to crystallinity and increases in direct proportion with crystallinity (Htoon et al., 2009). Since thermal processing has an impact on crystallinity in starch and rate of crystallization in starch depending on water content (Jouppila et al., 1998), this spectral difference could be due to molecular order changing in starch which might be occurred during the drying process. The peak around 1238 cm$^{-1}$ was sourced from –C–O stretching and –CH$_2$ bending vibrations in fats. The band at 1398 cm$^{-1}$ was due to C–C–H and C–O–H deformation in carbohydrates. Absorption at 1540 cm$^{-1}$ was sourced from the Amide II band, which is due to C–N stretching vibrations in combination with N–H bending vibrations in proteins (Guo et al., 2013). The peak around 1633 cm$^{-1}$ was sourced from the Amide I band of ß-sheet structures due to C=O stretching vibrations (Barth, 2007; Bunaciu et al., 2014). This spectral difference might be caused by the difference in protein contents of pistachio nuts and green peas. Absorption around 1741 cm$^{-1}$ was sourced from C=O stretching vibrations of fatty acid esters in fats, while 2854 and 2923 cm$^{-1}$ were originated from C–H asymmetric and symmetric stretching vibrations of long-chain fatty acids. This difference between pure samples could be related to the fact that pistachio nuts contain more long-chain fatty acids than green peas (Kashaninejad, 2011; Tulbek, 2017).

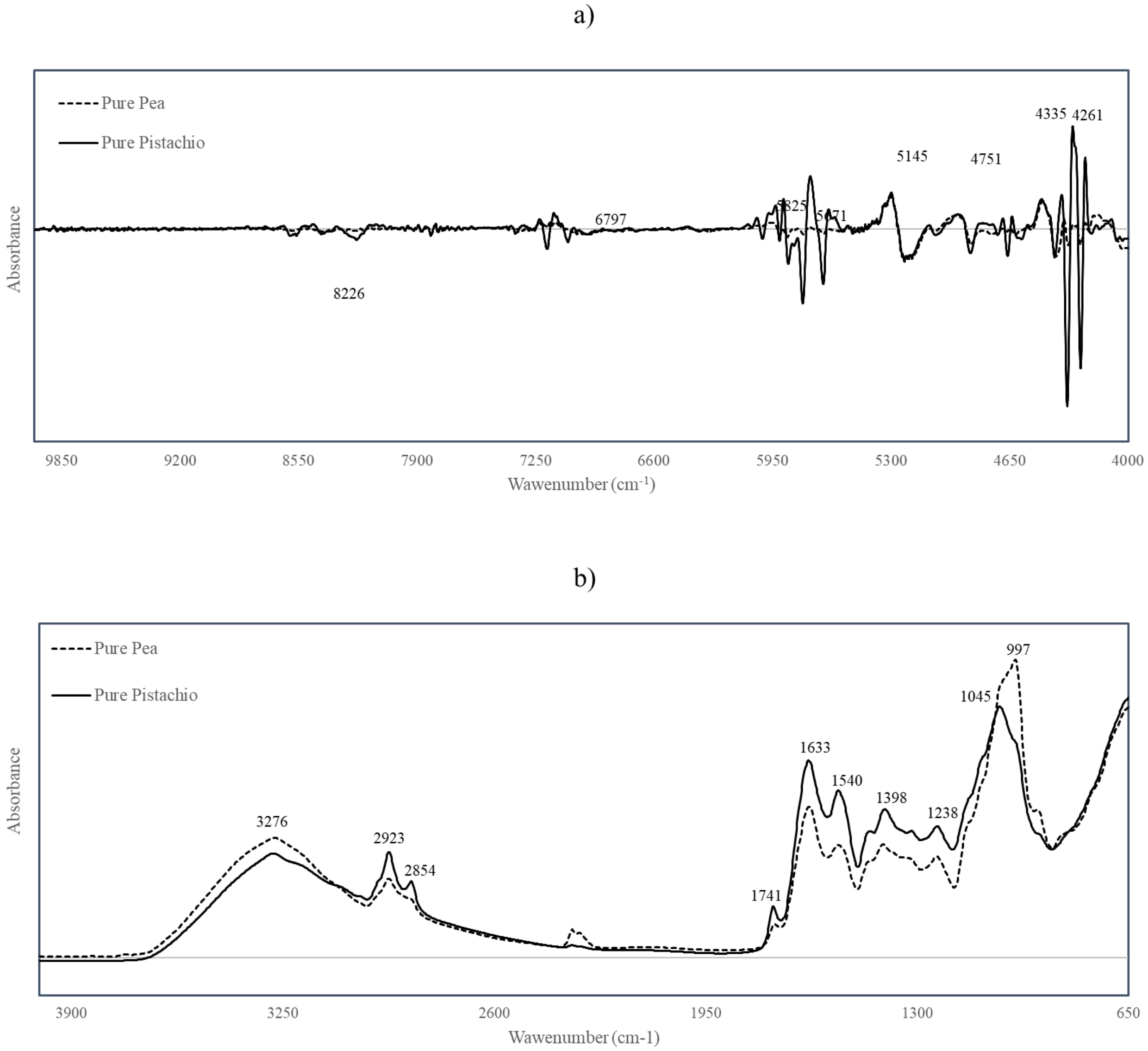

**Figure 1.** Average of the collected spectra using NIR and MIR-ATR for pure dried green pea seeds and pure ground pistachio nut samples (a: NIR spectra (second derivative transformed (Savitzky-Golay second-order polynomial filter with a 25-point window); b: MIR-ATR spectra)

### Partial Least-Squares Regression (PLSR) Models

During the PLSR model developments, combinations of various preprocessing options and different transformations have been tested to improve the linearity between the adulteration levels and the IR spectra (Christie 1983). As pretreatments, MSC (multiplicative scatter correction) method yielded the best results to the NIR spectra decreasing the spectral scattering caused by different particle sizes within the samples. As for the MIR spectra, second derivative transformation (Savitzky-Golay second-order polynomial filter with 25-point window) and normalization (Savitzky-Golay second-order polynomial filter with 35-point window) procedures decreasing the spectral scattering and fixing baseline were found to be superior.

For both NIR and MIR-ATR, calibration models were developed first, and then models were verified using cross-validation and external sample set. The ratio of dried green pea seeds in the samples belonging to the calibration set varied between 0% and 50%. In comparison, the range in the validation set was 3% and 47% (Table 2), exhibiting similarities between calibration and validations sets as expected. In PLSR model development, the best performance was obtained using part of the NIR (8219-4000 cm-1) and MIR-ATR (3060-650 cm-1). The optimum number of PLS-factors was 4 in both models.

**Table 2.** The statistical parameters of calibration and validation sets used in the development of the PLSR models

| Parameter | Sample Set | Amount of Sample | Minimum (%) | Maximum (%) | Average (%) | Standard Deviation (%) |
|---|---|---|---|---|---|---|
| **The Ratio of Green Pea (%)** | **Calibration** | 51 | 0 | 50 | 25 | 14.9 |
| | **Validation** | 12 | 3 | 47 | 27 | 14.4 |

The statistical performance of the PLRS models is given in Table 3. SEP value was used to measure the uncertainty of the prediction, which is the measurement of the difference between predicted and reference values. SEP and rPred values of NIR models were determined as 2.55% and 0.99, respectively. However, these values of MIR-ATR models were 9.14% and 0.80, respectively, and considered as unsatisfactory in performance. Higher rPred value and lower SEP value showed that the model based on NIRS was suitable for the detection of the green pea adulteration in pistachio nuts. Furthermore, RPD value is very significant and notable statistical data that shows the performance of PLSR models. The RPD values of PLSR models based on NIR and MIR-ATR techniques were determined as 5.7 and 1.6, respectively. Since the RPD value ranging from 5.0 to 6.4 is taken into consideration as a good and can be used for repeated analysis and quality control application, obtained RPD result of the NIR model indicated a good model for prediction of green pea adulteration in pistachio nuts.

**Table 3.** Statistical performance of PLSR models developed for detection of dried green pea seed ratio (%) in binary mixtures

| | **Spectral region ($cm^{-1}$)** | **Pre-processing method** | **Sample in calibration set (n)** | **Sample in validation set (n)** | **Number of Factors** | **SEC (%)** | **SECV (%)** | **SEP (%)** | **rCal** | **rCV** | **rPred** | **RPD** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| NIR | 8219 - 4000 | MSC (Multiplicative Scatter Correction) | 51 | 12 | 4 | 1.57 | 1.67 | 2.55 | 0.99 | 0.99 | 0.99 | 5.7 |
| MIR-ATR | 3060 - 650 | Normalization and 2nd order derivative | 51 | 12 | 4 | 6.14 | 6.77 | 9.14 | 0.92 | 0.89 | 0.8 | 1.6 |

SEC (standard error of calibration), SECV (standard error of cross-validation), SEP (standard error of prediction), rCal (correlation coefficient of calibration), rCV (correlation coefficient of cross-validation), rPred (correlation coefficient of prediction), RPD (residual predictive deviation)

PLSR correlation graphs of NIR and MIR-ATR spectra developed to predict green pea adulteration (%) in pistachio nuts are shown in Fig 2 and Fig 3, respectively. The coefficients of determination ($R^2$) regarding calibration and validation models of NIR were determined as 0.98 and 0.97, respectively. Likewise, for MIR-ATR, the $R^2$ value of calibration and validation sets were determined as 0.79 and 0.65, respectively. The closeness of the $R^2$ value of calibration and validation sets is associated with the success of the regression model (Cuadrado et al., 2005). The $R^2$ value that is higher than 0.90 is evaluated as an excellent prediction, and the $R^2$ value between 0.70-0.90 is evaluated as a good precision (Cuadrado et al., 2005). According to these results, based on the $R^2$ values, the prediction performance of NIR spectroscopy can be considered as excellent precision. In contrast, it can be considered as good precision for MIR-ATR spectroscopy. NIR spectral collections were made using about 5 g of samples on about glass petri dish of approximately 5 cm in diameter. Additionally, the sample cup was rotated continuously throughout the spectra collection for more homogenous spectra collection. Furthermore, the NIR region has higher energy than MIR-ATR, and this leads to a drastically longer penetration depth into the sample. These facts may have contributed to the superior performance of the NIR spectroscopy. On the other hand, a minimum amount of sample was placed to cover 1.5 mm crystal in diameter, and the MIR-ATR can penetrate shorter distances significantly (30-50 µm) into the sample on the crystal and collect the information mostly from the surface (Pu et al. 2019). Accordingly, the pressure applied to provide good contact between the crystal and the sample during the MIR-ATR spectra collection may have caused oil of ground pistachio to separate. Therefore, solid samples such as the binary mixtures in this study may have contained some heterogeneity within the sample placed on the crystal contributing to the poor performance of the MIR-based PLSR model.

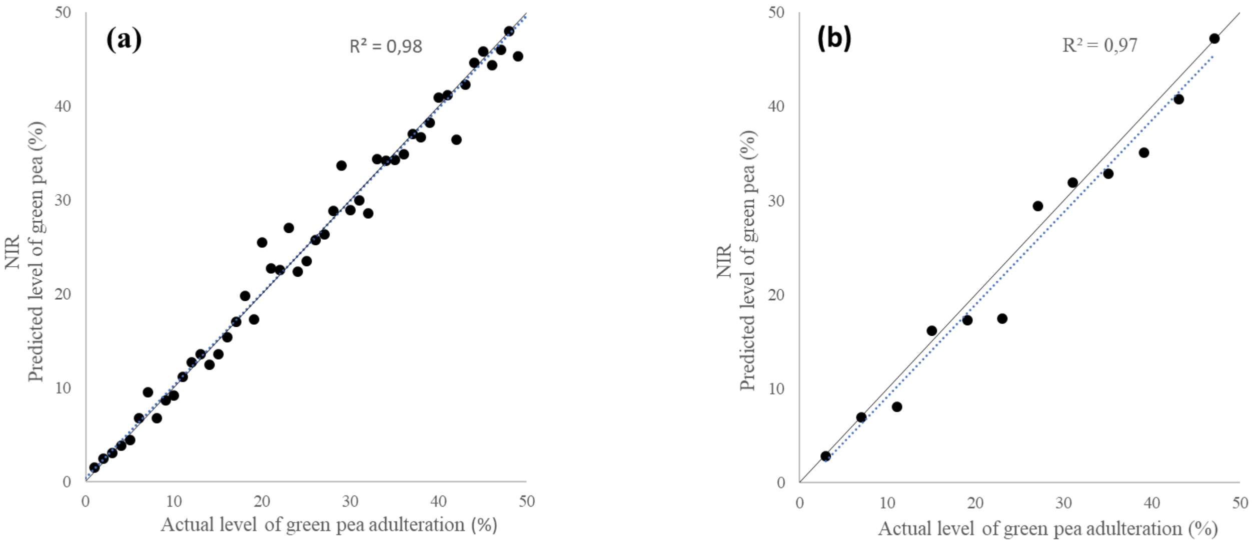

**Figure 2.** PLSR correlation graph based on NIR spectra of the adulteration level of the dried green pea seeds (%) in binary mixtures (a: calibration set of samples; b: validation set of samples)

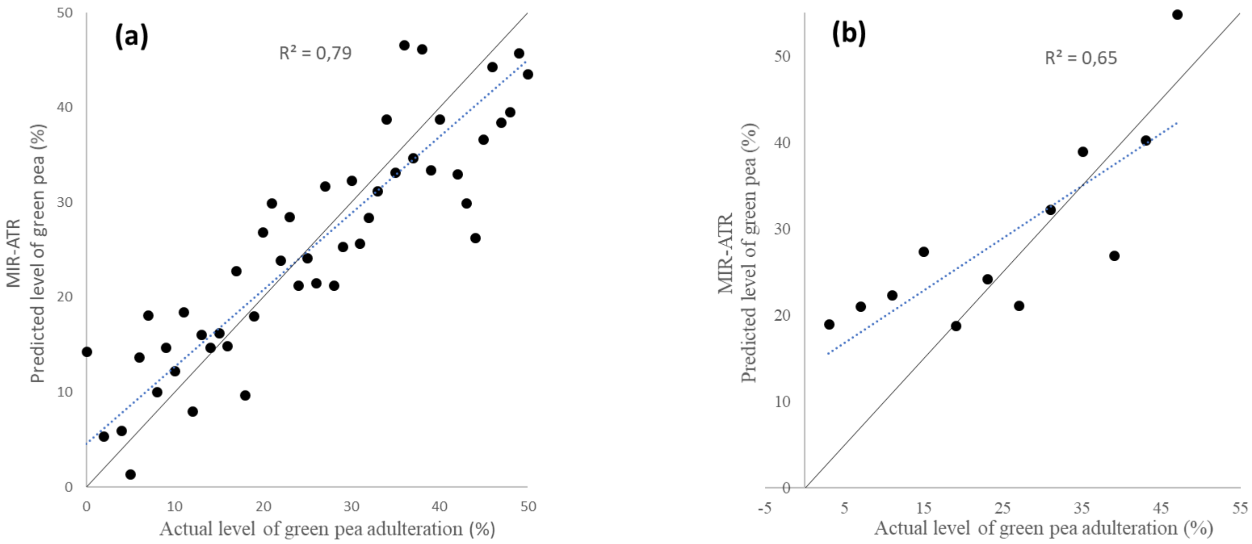

**Figure 3.** PLSR correlation graph based on MIR-ATR spectra of the adulteration level (%) of the dried green pea seeds in binary mixtures (a: calibration set of samples; b: validation set of samples)

## Conclusions

In this study, the green pea adulteration level was aimed to be determined in the ground pistachio nuts. Statistical performances of models developed by using spectral data obtained in NIR and MIR-ATR methods combined with chemometrics have been demonstrated to make objective evaluations. Overall, NIR spectroscopy performed drastically superior compared to MIR-ATR. MIR-ATR was found insufficient in its predictive performance. The results obtained in this study suggest that NIR spectroscopy can be used successfully in routine quality control practices of ground pistachio nuts for possible green pea adulteration.

## Author Contributions

Riza Temizkan contributed to the analysis of the results and drafted the manuscript. Muhammed Ali Dogan carried out the experiment and helped to draft the manuscript. Orhan Atakan carried out the experiment and helped to draft the manuscript. Burak Alptug Nazlım carried out the experiment, made peak identifications, and helped to draft the manuscript. Huseyin Ayvaz design and supervised the study.

## Conflict of Interest

The authors declare that there is no conflict of interest.

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