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Research Article

Rapid detection of walnut and pumpkin oil adulteration using Raman spectroscopy and partial least square methodology

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Abstract

The purpose of this study is to develop a statistical method, based on Raman spectroscopy results, to quickly identify the adulteration of pumpkin and walnut oils. For this purpose, pure pumpkin and walnut oils from Cluj County, Romania were studied with Raman techniques. They were adulterated with sunflower oil at 14 levels of concentration, ranging from 2.5 to 50%. The areas under the significant peaks were quantified and compared. A statistical method using the partial least square methodology was developed and used as a prediction tool in order to establish the adulteration percentage for pumpkin and walnut oils. 4 components were used to model the equation, the peak areas from ~1264, ~1300, ~1441 and respectively ~1659 cm-1. The final model equations take into account only the peak areas that had a high impact on the prediction values, statistically proven using the p-value. The level of prediction obtained with the final model equation was ≥ 95%.

Keywords: adulteration; partial least square methodology; Raman; pumpkin oil; walnut oils; spectroscopy; rapid detection

Introduction

Vegetable oils are liquid or solid fats extracted from seeds, cereal grains, nut and fruits with a composition based on a mixture of triacylglycerols, diacylglycerols, tocopherols, tocotrienols, phytosterols esters and phytosterols (Hammond, 2003). Vegetable oils can be traced back in time, starting with olive oil from 6000 BC in Israel (McMathis, 2015), palm and coconut oils from 4000 BC in Egypt (Roccisano *et al.*, 2016) and soybean oil from 1000 AC in China (https://www.soyinfocenter.com). Nowadays, they are intensively used in food processing, as fuel, in several home products and personal care products. Due to their wide usage, the global production has increased by approximately five times from 2007 to 2020 (https://www.statista.com).

In human consumption, vegetable oils are used for cooking, frying, in salads and as components of several types of processed foods. The consumption of vegetable oils is encouraged by the modern medicine which shows that the absorption of nutrients and vitamins depends on the functioning of hormonal processes and the consumption of fats (Conlon *et al.,* 2012; Roccisano *et al.*, 2016). Also, the fatty acids from vegetable oils have an important role in therapeutic and prophylactic prevention of many diseases, fetal neurological and reproductive development, optimal cardiovascular and endocrine functioning of adult humans and are now known to have anticancer potential (Hornstra *et al.*, 1995; Breslow *et al.*, 2006; Roccisano *et al.*, 2016; Kumar

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et al., 2016). Compared with animal fats, the usage of vegetable oils in food manufacturing is considered to be a much healthier alternative then animal fats due to their higher content of unsaturated fatty acids (https://www.statista.com).

In recent years two new types of vegetable oils have received special attention due to their benefits for human health (Elfiky *et al.*, 2012; Nederal *et al.*, 2014; Nishimura *et al.*, 2014; Seif, 2014; Abou-Zeid *et al.*, 2018; Fawzy *et al.*, 2018; Wong *et al.*, 2019).

Pumpkin oil is obtained from pumpkin (*Curcubita pepo* L), a leafy green vegetable native from North America, but cultivated now also in Europe, Asia and Africa (Nederal *et al.*, 2014; Nishimura *et al.*, 2014). Pumpkin seeds are an important source of vitamins, fatty acids, various antioxidants, amino acids and fibres (Nishimura *et al.*, 2014; Seif, 2014; Fawzy *et al.*, 2018; Wong *et al.*, 2019). Due to the composition of pumpkin seeds, pumpkin oil is used in human consumption for its nutritional value but was also tested in medicine as adjuvant treatment in: urinary dysfunction (Nishimura *et al.*, 2014), protection against alcohol induced hepatotoxicity and oxidative stress (Seif, 2014), alleviation of the adverse effects of bisphenol A in human (Fawzy *et al.*, 2018), reducing both brachial and central blood pressure as well as wave reflection in postmenopausal women with elevated blood pressure (Wong *et al.*, 2019), protection against the cytotoxicity and genotoxicity induced by azathioprine (Elfiky *et al.*, 2012), reducing the oxidative stress, apoptosis and toxic impacts on metabolizing enzymes gene expression induced by emamectin (Abou-Zeid *et al.*, 2018).

Walnut oil is obtained from walnut tree (*Juglans regia* L) which is, native from the mountain region of Central Asia and has become the most widespread tree nut in the world. According to the Food and Agriculture Organization over 1000000 metric tons of walnut oil are produced annually as follows: China (350000 tons), United States of America (210000 tons), Turkey (114000 tons), Iran (82000 tons), Europe (250000 tons) (http://www.fao.org). Walnut oil is composed mainly of polyunsaturated fatty acids (especially linoleic acid), tocopherols, proteins, minerals, phospholipids, sphingolipids, sterols, hydrocarbons and volatile compounds (Martínez *et al.*, 2010; Rabadán *et al.*, 2018). Its composition has important benefits on human health: it improves blood lipoprotein profile, shows anticancer and antiatherogenic effect, and contributes to the regulation of immunological activity and inflammatory response (Rabadán *et al.*, 2018), has high antioxidant activity and can be considered as cancer chemo-preventive (Negi *et al.*, 2011). Due to its high price, walnut oil is adulterated with cheaper edible oil such as soybean or sunflower oil (Li *et al.*, 2015).

The adulteration of edible oils is the process of adding cheaper and inferior oils that affect the quality of the product in order to reduce the fabrication costs. The adulterated oils can be a real threat to human health, such as the case of Spanish olive oil syndrome were nonedible rapeseed oil was sold as edible rapeseed oil and even as olive oil or the adulteration of mustard oil with poisonous argemone oil (Azadmard-Damirchi and Torbati, 2015). Nowadays, the adulteration of oils became more discreet so their values remain within the limits established by regulations (Azadmard-Damirchi and Torbati, 2015; Abbas and Baeten, 2016). In this situation it is imperative to develop sensitive analytical techniques to establish the purity of the oils. The most sensitive methods which were proposed are: nuclear magnetic resonance (NMR) (Hatzakis, 2013; Parker *et al.*, 2014; Nam *et al.*, 2014; Shi *et al.*, 2018), near-infrared spectroscopy (NIR) (Chen *et al.*, 2012; Farres *et al.*, 2019; Yuan *et al.*, 2020), FT-infrared spectroscopy (FT-IR) (Man *et al.*, 2011; Man and Rohman, 2013) and Raman spectroscopy (Wei *et al.*, 2013; Velioglu *et al.*, 2016; Meenu *et al.*, 2019). Raman spectroscopy is a quick and sensitive method which does not need any sample preparation in order to identify the adulteration of oils.

The purpose of this study is to develop quick and easy methods to determine the adulteration of pumpkin and walnut oils using portable Raman equipment and to create a pattern of behaviour of these oils when adulterated using the partial least squares (PLS) statistic method for the development of the model equation.

Materials and Methods

Sample collection and preparation

Pure pumpkin, walnut and sunflower oils of Romanian provenience were acquired from producers of edibles oils from Cluj County. The pumpkin and walnut oils were adulterated with 14 levels of concentration of sunflower oil ranging from 2.5 to 50%.

Raman analysis

All the oils were investigated with Rigaku PROGENY portable Raman using the following parameters: laser frequency: 1064 nm; laser power: 200 mW; exposure time: 10,000 ms; spectral range: 200-2000 cm-1. All Raman analyses were performed in triplicate at room temperature using 4 ml of each sample.

Data processing

The raw data were processed using multiple peak function from Origin 8.5 (OriginLab, USA).

Statistical analysis

For statistical analysis the data were processed using Minitab 17 software. Partial least squares (PLS) regression was used to model the equation of adulterated oil concentration. To perform PLS, Minitab uses the nonlinear iterative partial least squares (NIPALS) algorithm developed by Herman Wold. The algorithm reduces the number of predictors using a technique similar to principal components analysis to extract a set of components that describes maximum correlation between the predictors and response variables (*https://support.minitab.com*).

Validation of model

The model equation was tested using adulterated oil samples to see if the degree of adulteration prediction is equal or higher than 95%.

Results

Raman analysis

Raman spectra of investigated pumpkin, walnut and sunflower oils are presented in Figure 1.

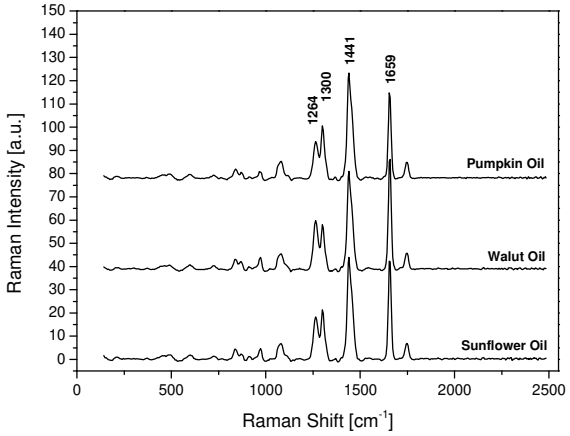

Figure 1. Raman spectra of investigated pumpkin, walnut and sunflower oils

The obtained peaks and their assignments (Meenu *et al.*, 2019) are presented in Table 1. The Raman peaks from ~1659 cm^{-1} and ~1264 cm^{-1} are assigned to vibration peaks of cis (C-C) and cis (C=H) of unsaturated fatty acids while the peaks from at ~1441 cm^{-1} and ~1300 cm^{-1} are assigned to scissoring vibrations and twisting vibrations of methylene (Li *et al.*, 2018). Peak areas are presented in Table 2. For all investigated oils, the highest peak area is at ~1441 cm^{-1} corresponding to scissoring vibrations of methylene. The lowest peak area is at ~1264 cm^{-1} corresponding to vibration peaks of cis (C=H) of unsaturated fatty acid. Walnut and sunflower oils have the highest peak areas assigned to vibrations of unsaturated fatty acids groups while pumpkin oil have the highest peak areas assigned to vibrations of methylene groups.

Tabel 1. Main Raman peaks and their assignment (Li *et al.*, 2018)

No	Wavenumber (cm^{-1})	Assignment
1	~1264	δip(=C—H) Unconjugated cis (RHC=CHR)
2	~1300	δγ(C-H) of Methylene (CH2)
3	~1441	δγ(C-H) of Methylene (CH2)
4	~1659	ν(C=C) of unsaturated banding (cis RHC=CHR)

Table 2. Peak areas of investigated pumpkin, walnut and sunflower oils

Oil type/ Wavenumber (cm^{-1})	Area [a.u.]			
	~1264	~1300	~1441	~1659
Pumpkin	468002±2.7%	539749±4.1 %	1481990±5.1%	664080±2.9%
Walnut	539254±3.5 %	506907±2.8 %	1425604±2.9%	788539±2.8%
Sunflower	539248±3.8 %	506911±3.7 %	1425610±3.8%	788540±4.6%

The peak areas of adulterated pumpkin and walnut oils are presented in Figure 2. For pumpkin oil a small decrease of ~1659 cm^{-1} and ~1264 cm^{-1} peak areas, an insignificant decrease of ~1300 cm^{-1} peak area, and a significant decrease of ~1441 cm^{-1} peak area could be observed. For walnut oil an increase of ~1264 cm^{-1}, ~1300 cm^{-1} and ~1441 cm^{-1} peak areas and an important decrease of ~1659 cm^{-1} peak area could be observed.

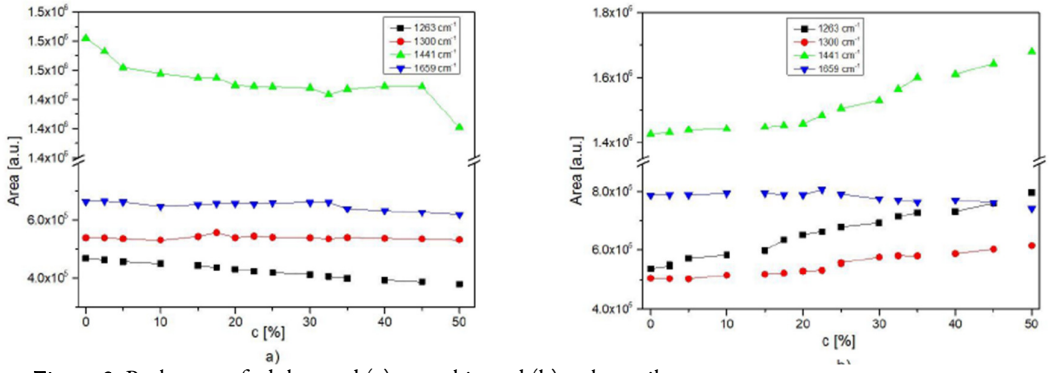

Figure 2. Peak areas of adulterated (a) pumpkin and (b) walnut oils

Although the data presented in Figure 2 show ascending or descending trends of pumpkin and walnut oils peaks varying together in line with the percentage of adulteration with sunflower oi, a conclusive behaviour cannot be drawn. A supplementary analysis of data is mandatory.

Partial least squares (PLS) regression

The peaks area of pumpkin and walnut oils obtained from Raman analysis for every level of adulteration are presented in Table 3.

Table 3. Peaks area of pumpkin and walnut oils obtained from Raman analysis for the adulterated oils

c (%)/ Wavenumber (cm^{-1})	Area [a.u.]							
	~1264		~1300		~1441		~1659	
	PO	WO	PO	WO	PO	WO	PO	WO
0	484806 ±2.6%	628959 ±3.7%	605008 ±4.5%	505902 ±2.4%	1581677 ±5.4%	1476560 ±2.7%	475263 ±2.4	606521 ±2.7
2.5	484028 ±2.9%	631976 ±3.0%	601322 ±4.3%	507804 ±2.4%	1571197 ±4.8%	1478999 ±2.9%	475281 ±2.9	594085 ±2.9
5	483740 ±2.4%	652526 ±2.9%	586626 ±3.9%	513614 ±2.9%	1574645 ±5.1%	1482811 ±2.4	470911 ±3.1	593807 ±3.1
10	481608 ±2.7%	658709 ±2.7%	574388 ±4.1%	519347 ±3.1%	1544332 ±5.0%	1482365 ±2.1	457473 ±3.2	590837 ±2.7
15	480428 ±2.4%	668984 ± 2.6%	580635 ±3.9%	523667 ± 3.2%	1537402 ±5.1%	1484014 ±2.8	458978 ±3.3	585064 ±3.1
17.5	478094 ±2.6%	702826 ±2.5%	588239 ±4.5%	528680 ±3.3%	1533784 ±5.4%	1485834 ±3.1	458760 ±3.4	574410 ±3.2
20	476568 ±2.4%	713699 ±3.4%	563430 ±4.2%	536984 ±3.4%	1524396 ±4.7%	1487071 ±3.2	457001 ±3.1	567783 ±3.4
22.5	475330 ±2.5%	718866 ±3.5%	562304 ±3.8%	540892 ±3.5%	1519993 ±3.9%	1509810 ±3.4	452975 ±2.9	574354 ±3.1
25	474667 ±2.5%	732367 ±3.1%	551986 ±3.9%	565187 ±3.6%	1515965 ±3.9%	1529252 ±2.9	452975 ±2.2	557686 ±3.2
30	472622 ±2.7%	739416 ±3.2%	544152 ±4.1%	585060 ±3.7%	1511493 ±5.1%	1550377 ±2.1	451071 ±2.3	540572 ±3.1
32.5	470894 ±2.9%	756263 ±2.7%	538622 ±4.2%	591538 ±3.1%	1503129 ±5.2%	1581395 ±2.3	448950 ±2.4	531774 ±2.4
35	469627 ±2.8%	761580 ±2.6%	537086 ±4.3%	592515 ±3.8%	1535934 ±5.1%	1614131 ±2.9	431776 ±2.9	521769 ±2.1
40	468330 ±2.5%	759250 ±2.5%	528688 +4.5%	601539 ±3.4%	1501559 ±4.7%	1620210 ±2.8	424703 ±2.5	520859 ±2.2
45	466314 ±2.2%	780307 ±3.4%	520982 ±4.0%	617967 ±3.7%	1497872 ±4.3%	1649854 ±2.5	418682 ±2.4	510409 ±2.3
50	462858 ±2.3%	810864 ±3.5%	512917 ±4.2%	632081 ±3.9%	1456499 ±4.1%	1682177 ±2.1	411283 ±2.7	491643 ±2.7
Linear correlation coefficient	-0.996	0.985	-0.976	0.979	-0.925	0.938	-0.961	-0.981

1 PO – pumpkin oil
2 WO – walnut oil

The linear correlation coefficient values were from -0.925 to - 0.996 for the negate correlation and between 0.938 to 0.985 for the positive correlation which proves the strong connection between degrees of adulteration and the area at the selected wavelengths.

Four components where used to model the equation, the areas of the peaks at ~1264 cm^{-1}, ~1300 cm^{-1}, ~1441 cm^{-1} and ~1659 cm^{-1}.

PLS for the walnut oil

Analysis of variance was applied on the data obtained for the walnut oil. The results are presented in Table 4.

Table 4. Analysis of variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Regression	4	3284.11	821.027	166.80	0.000
~1264 cm^{-1}	1	52.69	52.690	10.70	0.008
~1300 cm^{-1}	1	3.00	2.999	0.61	0.453
~1441 cm^{-1}	1	1.40	1.403	0.29	0.605
~1659 cm^{-1}	1	0.14	0.137	0.03	0.871
Error	10	49.22	4.922		
Total	14	3333.33			

The degree of freedom (DF) represents the number of independent comparisons between the elements of an observation quantity or the number of values that can be chosen arbitrarily within a specification. It is used to estimate the values of unknown population parameters. Adjusted sums of squares (Adj SS) are measures of variation for different components of the model. Adjusted mean squares measure (Adj MS) how much variation a term or a model explains, assuming that all other terms are in the model, regardless of the order they were entered. Minitab uses the adjusted mean squares to calculate the p-value for a term (www.statsdirect.com; https://support.minitab.com; Dodge, 2010). The F-value is used to determine whether the term is associated with the response. Minitab uses the F-value to calculate the p-value, which is used to make a decision about the statistical significance of the terms and model. The P-value is equal to the area under the distribution curve, according to the statistical value of the test. The values should be lower than 0.05. Lower p-values provide stronger evidence against the null hypothesis. For ~1300 cm^{-1}, ~1441 cm^{-1} and ~1659 cm^{-1} the p-value is high which means that they have little impact. For ~1264 cm^{-1} the p-values is lower than 0.05, 0.008 respectively.

R-sq is the percentage of variation in the response that is explained by the model. It is calculated as 1 minus the ratio of the error sum of squares to the total sum of squares. Thus, R-sq indicates how well a model fits the data. In order to vary the model, the R-sq was calculated. (Table 5). The closes R-sq of model is to 100%, the better the fit between the input variables and the result. Predicted R-sq indicates how well each calculated model predicts the response. Predicted R-sq is calculated by systematically removing each observation from the data set, estimating the regression equation, and determining how well the model predicts the removed observation (https://support.minitab.com; Dodge, 2010).

Table 5. Model summary

S	R-sq	R-sq (ad)	R-sq (pred)
2.21864	98.52%	97.93%	97.29%

The R-sq (pred) value for walnut oils is 97.29, which means that the probability of the model predicting the write adulteration concentration is very high.

The correlation between the different variables for the adulteration of walnut oil is presented in Figure 3.

From the graphic (Figure 3) we can see that peaks at ~1264 cm^{-1}, ~1300 cm^{-1} and ~1441 cm^{-1} have a positive correlation with the adulteration concentration of walnut oil, while the peak at ~1659 cm^{-1} has a negative correlation.

The regression equations for the adulteration of walnut oils are: $Concentration\,(\%) = -163 + 0.000169 \cdot X1 + 0.000081 \cdot X2 + 0.000023 \cdot X3 - 0.000027 \cdot X4$ (1)

where:

X1 – peak area at ~1264 cm^{-1}

X2 – peak area at ~1300 cm^{-1}

X3 – peak area at ~1441 cm^{-1}

X4 – peak area at ~1659 cm^{-1}

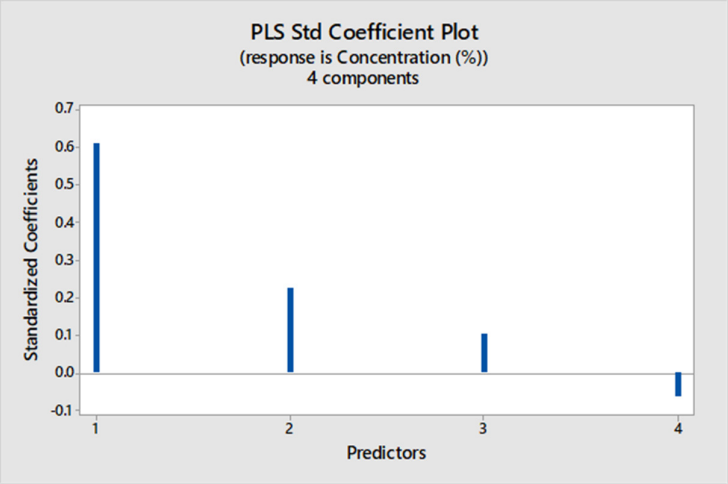

Figure 3. Projected scatterplot of the unstandardized regression coefficients for the adulteration of walnut oils

Figure 4 shows residual normal plot for the Raman analysis obtained for walnut oil. The points follow a straight line which proves that there are no unusual observations.

The final model equation does not contain all variables because the p-value has proven that some of them are not relevant. Figure 5 shows that ~1264 cm^{-1} has the highest impact while, the peaks at ~1441 cm^{-1} and ~1659 cm^{-1} do not help to explain the additional variation of the adulteration concentration.

The final model equation is:

$$Wallnut\ oil\ adulteration\ \% = -175.43 + 0.000164 \cdot X1 + 0.000147 \cdot X2 \quad (2)$$

where:

X1 – peak area at ~1264 cm^{-1}

X2 – peak area at ~1300 cm^{-1}

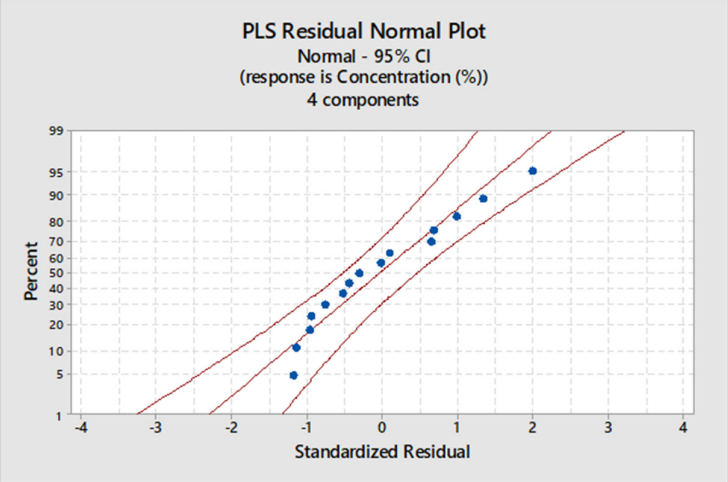

Figure 4. PLS residual for walnut oil

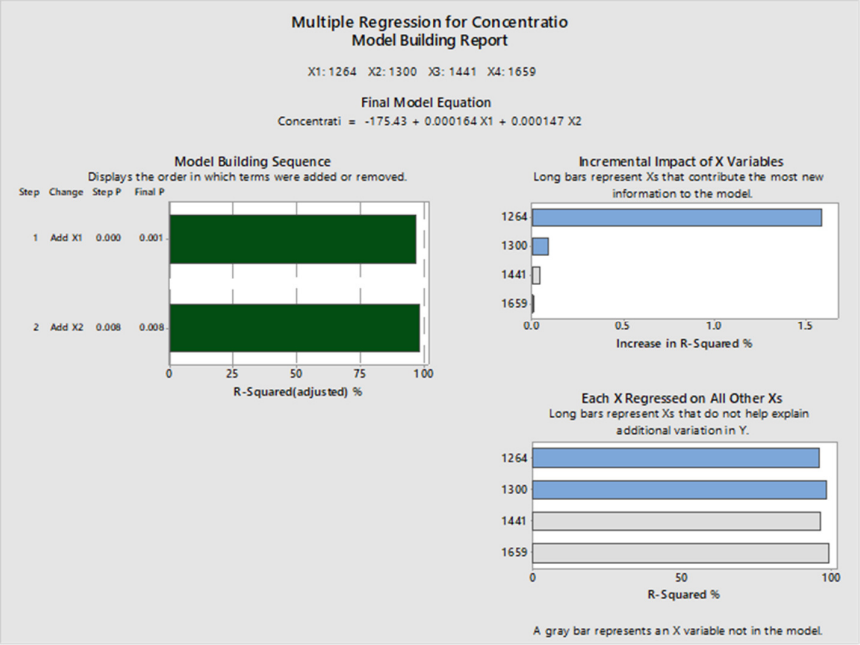

Figure 5. Model building report for adulteration of walnut oil

PLS for the pumpkin oil

Analysis of variance was applied on the data obtained for the pumpkin oil. The results are presented in Table 6.

Table 6. Analysis of variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Regression	4	3314.67	828.667	444.01	0.000
~1264 cm^{-1}	1	73.16	73.161	39.20	0.000
~1300 cm^{-1}	1	2.28	2.279	1.22	0.295
~1441 cm^{-1}	1	0.92	0.919	0.49	0.499
~1659 cm^{-1}	1	2.52	2.516	1.35	0.273
Error	10	18.66	1.866		
Total	14	3333.33			

For ~1300 $cm^{-}1$, ~1441 cm^{-1} and ~1659 cm^{-1} the p-value is high which means that they have little impact. For ~1264 cm^{-1} the p-values is 0.000 which means that this factor has a very important impact.

In order to verify the model the R-sq was calculated (Table 7).

Table 7. Model summary

S	R-sq	R-sq (ad)	R-sq (pred)
1.36614	99.44%	99.22%	98.64%

The R-sq predicted for pumpkin oils has a value of 98.64%.

The regression equations for the adulteration of pumpkin oils are: $\text{Concentration (\%)} = -163 + 0.000169 \cdot X1 + 0.000081 \cdot X2 + 0.000023 \cdot X3 - 0.000027 \cdot X4$ (3)

where:

X1 – peak area at ~1264 cm^{-1}

X2 – peak area at ~1300 cm^{-1}

X3 – peak area at ~1441 cm^{-1}

X4 – peak area at ~1659 cm^{-1}

Figure 6 shows residual normal plot for the Raman analysis obtained for pumpkin oil. The points follow a straight line which prove that there are no unusual observations.

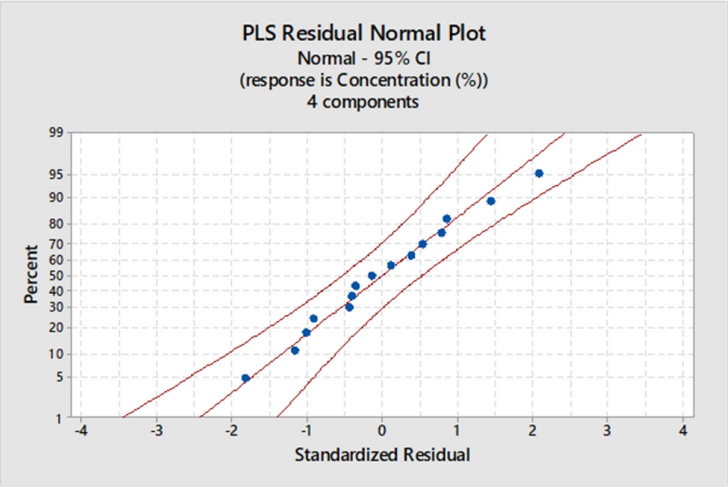

Figure 6. PLS residual for pumpkin oil

The correlation between the different variables on the adulteration of pumpkin oil is presented in Figure 7.

There is a negative correlation on the adulteration concentration for all 4 peaks (~1264 cm^{-1}, ~1300 cm^{-1}, ~1441 cm^{-1} and ~1659 cm^{-1}).

The final model equation does not contain all variables because the p-value has proven that some of them are not relevant. Figure 8 shows that peak at ~1264 cm^{-1} has the highest impact while peaks at ~1300 cm^{-1}, ~1441 cm^{-1} and ~1659 cm^{-1} do not help explain additional variation of the adulteration concentration.

The final model equation is:

$$Wallnot\ oil\ adulteration\ \% = 1090.4 - 0.002245 \cdot X1 \quad (4)$$

where: X1 - area of the peak at ~1264 cm^{-1}.

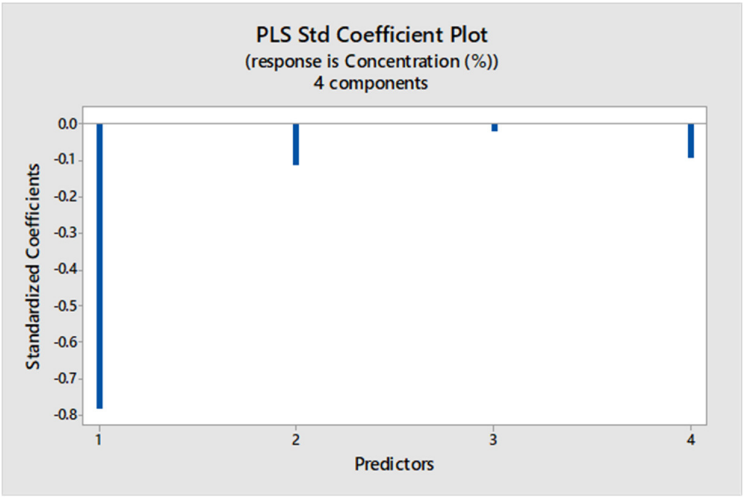

Figure 7. Projected scatterplot of the unstandardized regression coefficients for the adulteration of pumpkin oils

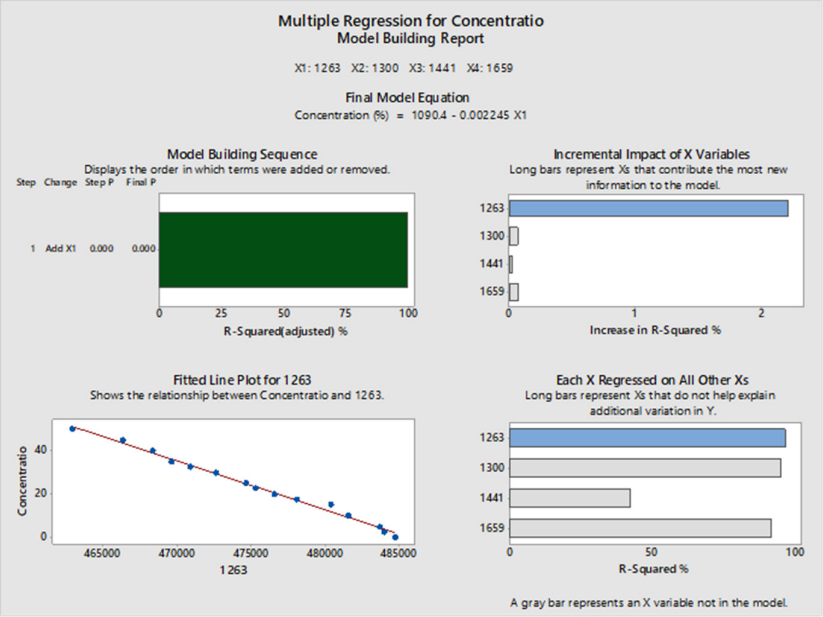

Figure 8. Model building report for the adulteration of pumpkin oil

In order to use the equation for the prediction of adulterated oils, the degree of prediction must be ≥ 95 %. The final prediction equation was tested for each oil type to see if it can correctly predict the percentage of adulteration. The obtained results are presented in Table 8.

Table 8. Prediction equation for different oils samples

No.	Sample type	Adulteration (%)	Predicted value (%)	The degree of adulteration prediction (%)	Remarks
1.	Walnut oil	5	4.8	96.0	Meets the criteria
2.		10	10.1	99.0	Meets the criteria
3.		15	15.4	97.3	Meets the criteria
4.		30	30.2	99.3	Meets the criteria
5.	Pumpkin oil	5	5.2	96.0	Meets the criteria
6.		10	10.5	95.0	Meets the criteria
7.		15	15.2	98.7	Meets the criteria
8.		30	30.7	97.7	Meets the criteria

Discussion and conclusions

A Raman portable spectrometer, which allows quick measurements on-site, was used to verify its sensitivity for testing the adulteration of walnut and pumpkin oils with sunflower oil. Different levels of concentration of sunflower oil, ranging from 2.5 to 50%, were used to obtain adulterated samples. Four peaks of Raman spectra were selected, based on literature (Velioglu *et al.*, 2016; Wei *et al.*, 2013; Meenu *et al.,* 2019; Li *et al.*, 2018). These peaks situated at ~1264 cm^{-1}, ~1300 cm^{-1}, ~1441 cm^{-1} and ~1659 cm^{-1} were slowly modified with the increase of the degree of adulteration. These changes were quantified by the calculation and graphical representation of Raman peak areas. In order to obtain complex data from Raman spectra, a PLS statistic method was used to develop a prediction equation. An equation model was obtained for each type of studied oil with a prediction degree ≥ 95 %. In the literature there are few studies available on adulteration of walnut and pumpkin oils using Raman spectroscopy but the data obtained were in good agreement with similar studies performed on other types of vegetal oils (Aykas *et al.,* 2019; Mendes *et al.,* 2015). These results confirm

that the Raman technique can be easily and successfully used in predicting the adulteration of pumpkin and walnut oils.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article. The authors read and approved the final manuscript.

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