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Assesing Pistachio Nut (*Pistacia vera L.*) Adulteration with Green Pea (*Pisum sativum L.*) by Untargeted Liquid Chromatography-(quadrupole-time of flight)-Mass Spectrometry Method and Chemometrics

Antep Fıstığı İçerisine Tağşiş Amacı ile Katılan Bezelyenin Sıvı Kromatografi-Uçuş Zamanlı Kütle Spektrometresi ile Kemometrik Olarak Belirlenmesi

Filiz ÇAVUŞ[1], Murat Faruk US[2], Nurcan A. GÜZELSOY[3]

[1] *Kimya Müh. Gıda ve Yem Kontrol Merkez Araştırma Enstitüsü Müdürlüğü-BURSA*
[2] *Dr.Kimyager. İstanbul Gıda Kontrol laboratuvar Müdürlüğü-İSTANBUL*
[3] *Gıda Yük. Müh. Gıda ve Yem Kontrol Merkez Araştırma Enstitüsü Müdürlüğü-BURSA*

Abstract

In this work, a method was developed for the detection of green pea adulteration in pistachio nut powder and related products using high-performance liquid chromatography quadrupole time-of-flight mass spectrometry (HPLC-QTOF MS). Pistachio nut is target for economically motivated adulteration due to its high price and causes economic losses to consumers. Electrospray ionization mass spectrometry ESI(-)-MS fingerprints were collected for twelve pistachio nut and green pea samples and mass spectra was found very distinctive for each pistachio nut and green pea samples. Polar components were extracted with methanol/water(1:1) solution. Traditional Turkish dessert "baklava" was used as a model food matrix. The detection of green pea adulteration was possible at a minimum level of 2,5% in baklava with ESI(-)-MS fingerprint. The ESI(-)-MS technique coupled with chemometric multivariate analysis; principal component (PCA) and hierarchical cluster analysis (HCA) was found a specific method to detect green pea marker ions in pistachio nut powder and related food products.

Keywords: Fingerprinting, Pistachio Nut, Green Pea, Principal Component Analysis; Hierarchical Cluster Analysis

Özet

Bu çalışmada antep fıstığı içerisine katılan bezelyenin yüksek performanslı sıvı kromatografisi uçuş zamanlı kütle spektrometresi (LC-QTOF MS) kullanılarak tespitine yönelik bir yöntem geliştirildi. Antep fıstığının ekonomik değerinin yüksek olması sebebi ile yapılan tağşişler tüketicilerin aldatılmasına ve üreticiler arasında haksız rekabete yol açmaktadır.

Çalışmada, elektrosprey iyonizasyon (ESI) tekniği ile Temel bileşenler analizi (Principal Component Analysis, PCA) ve hiyerarşik kümeleme analizi (Hierarchical Clusterin Analysis, HCA) kullanılarak antep fıstığına ve bezelyeye ait marker iyonlar tespit edildi. Gıda matriksi olarak geleneksel Türk tatlısı 'baklava' kullanıldı. Baklava örnekleri %1 ile %10 arasında farklı oranlarda bezelye karıştırılmış fıstık ile hazırlandı. ESI-MS parmak izi yöntemi ile fıstıklı baklava içerisinde %2,5 oranında bezelye tespit edildi.

Anahtar kelimeler: Parmak İzi, Antep Fıstığı, Bezelye, Temel Bileşen Analizi, Hiyerarşik Kümeleme Analizi

1. Introduction

Economically motivated food adulteration is intentional substitution of an authentic ingredient with a cheaper ingredient product without purchaser's knowledge for economic gain. Globalization has raised economically motivated adulteration as a key concern. There is an increasing number of food product recalls of adulteration each year which causes a loss of the consumer's trust in the food supply chain and economic consequences (Hussain and Dawson 2013); With an increasing public and regulatory attention on the quality and safety of food, there is a need to protect bothbuyers and sellers by developing analytical protocols that can beused to identify the adulteration.

Pistachio nut is one of the food which has been subjected to various adulterations due to its high price in several food products such as baklava (traditional dessert in Turkey), cake, ice-cream and chocolate (Koçak et al. 2016); Pistachio nut is an expensive nut species and it becomes a target for economically motivated adulteration.

Green pea (*Pisum sativum* L.) is commanly used in the adulteration of pistachio nut powder and related products due to its similarity of color and texture. Pistachio nut powder or granules are mainly used in the production of Turkish traditional dessert "baklava" which has an important role in Turkey export market. Turkey's Gaziantep baklava is the first Turkish product registered in the European Commission list of protected designations of origins and protected geographical indications. Therefore detection of pistachio nut adulteration is very important to protect consumer right and to provide food security in global level.

Near-infrared (NIR) spectroscopy (Winkler-Moser et al. 2015; Mabood at al. 2017), high-performance liquid chromatography (HPLC) and coupled techniques (Xu et al. 2017; Guijarro-Díez et al. 2017; Tay et al. 2013), gas chromatography (GC) and coupled techniques (Xu et al. 2015; Ko et al. 2014), fourier transform infrared (FT-IR) spectroscopy (Cebi et al. 2017; Petrakis and Polissiou 2017) and nuclear magnetic resonance (NMR) spectroscopy (Petrakis et al. 2015; Santos et al. 2016),methods have been widely used for detection of adulteration in the recent years. No studies have been reported until now to detect pistachio nut adulteration in baklava except Raman spectroscopy techniquedeveloped by Kocak (2016) to detect green pea in pistachio nut granules up to 20%w/w.

Electrospray ionization (ESI) is a soft ionization technique that is best applied to polar molecules, without the need of chemical derivatization or extraction from polar solutions (Catharino et al. 2005); Electrospray is especially useful for analyzing large biomolecules such as proteins, peptides, and oligonucleotides, but can also analyze smaller molecules like benzodiazepines and sulfated conjugates. ESI-MS with direct sample injection has also been demonstrated to be a powerful technique for fast fingerprint characterization of complex chemical mixtures (Souza et al. 2007, Koçak et al. 2017); such as beer (Araujo 2005), wine (Catharino et al. 2006; Cooper and Marshall 2001; Nunes Miranda et al. 2013) and vegetable oils (Wu et al. 2004);

The aim of the study was to develop an liquid chromatography mass spectrometry (LC-MS) based method using a quadrapole TOF MS instrument with electrospray ionization (ESI) unit for the detection of adulterations in pistachio nut powder. Turkish traditional dessert "baklava" was used as a model matrix to determine adulteration. Electrospray ionization mass spectrometry (ESI-MS) fingerprints data were handled by principal component analysis (PCA) and hierarchical cluster analysis (HCA) to be able to determine adulteration markers for routine quality control.

2. Materials and methods

Chemicals

Methanol of HPLC grade used for extraction of samples were supplied from Sigma Aldrich (St. Louis, MO, USA), deionized water was produced by a Milli-Q water purification system (Millipore, Bedford, MA, USA). Ammonium acetate from Sigma Aldrich (St. Louis, MO, USA) was used as mobile phase additives.

Material

A total of twenty four samples, twelve pistachio nuts (*Pistacia vera* L.) and twelve green peas (*Pisum sativum* L.) were purchased from local markets in different regions of Turkey (harvesting year 2014). Green pea and pistachio nut samples were dried in an oven at 700C for 12 h. After drying pistachio nuts and green pea were pulverized using a grinder. Ground green pea samples were mixed to pistachio nuts with five different weight rates (1, 2,5, 5, and 10 %(w:w)). Baklava was selected as a model matrix to determine adulteration markers in processed foods. Baklava samples were prepared by filling thin sheets of dough with differenet amounts of green pea and pistachio nut mixtures and baked at 2000C for 40 minutes. A sugar syrup was added after baking

Sample preparation

Green pea, pistachio nut and adulterated baklava samples containing different amounts of green pea and pistachio nut mixtures were finely ground in a mortar. Five grams of samples were extracted with a 10 mL methanol/water (50:50, v:v). After homogenization via ultra turrax for 2 min. at 11500 rpm, the samples were centrifuged for 10 min at 3500 rpm. 1.0 mL of supernatant was filtered through a 0.22 µm filter prior to injection

HPLC-QTOFMS

The HPLC-QTOFMS instrument used in this study was an Agilent 1260 infinity LC system (consisting of vacuum degasser, autosampler, binary pump and thermostatted column compartment) coupled to Agilent 6550 accurate mass QTOFMS with electrospray ionizationvia Jet Stream Technology (Agilent Technologies, Santa Clara, CA, USA). Chromatographic separation was performed using a Poroshell 120 EC reversed phase C18 analytical column (100 mm x 4,6 mm i.d., 2,7 µm particle size (Agilent Technologies, SantaClara, CA, USA). Column temperature was maintained at 400C. The injected sample volume was 5 µL in negative mode.

LC analyses with gradient elution were carried out with a flow rate 0,6 mL min-1by using a mobile phase of water containing 5 mM ammonium acetate (solvent A) and methanol (solvent B). The following gradient elution was carried out: from 0 to 0,5 min eluent B 5% ; from 0,5 to 28,0 min eluent B 95%; from 28,0 to 32,0 min eluent B 5%.

The mass spectrometric analysis was performed with an ESI source with Jet Stream technology using the following parameters: drying gas (N_2) flow rate: 14,0 L min-1; drying gas temperature: 1750C; nebulizer pressure:45 psig, sheath gas temperature:350 0C; sheath gas flow:11 L min-1; capillary voltage: 3500 V; nozzle voltage: 1000 V; skimmer voltage: 65 V; octapole RF:750 V; fragmentor voltage: 300 V. Accurate mass were recorded across the range 100-1500 m/z at an acquisition rate 2,0 spectra s-1. The instrument gave a typical resolution of 12,000 full widths at half maximum (FWHM). To guarantee mass accuracy, continuous internal calibration was carried out during analysis. For this reason, the reference solution was directly infused into the source; the purine (m/z121.0509) and protonated hexakis (1H,1H,3H-tetrafluoropropoxy) phosphazine (HP-921) (m/z 922.0098) signals were used to ensure accuracy and reproducibility.

Data Processing and statistical analysis

All HPLC-QTOFMS full single MS data including retention times (RT), mass to charge ratio and ion intensities were extracted by MassHunter Workstation software (version 3.01 Qualitative Analysis, Agilent Technologies, Santa Clara, CA, USA). The raw data set were initially analyzed by Molecular Features (MFs) extraction software for the detection of the compounds as molecular features (MFs) or entities characterized by RT, intensity in apex of chromatographic peak and accurate mass. Then the data was converted to compound exchange format file (cef files) for statistical analysis by the Mass Profiler Professional (MPP) software (version 2.0, Agilent Technologies, Santa Clara, CA, USA). In the next step, alignment of RT and m/z values was carried out across the sample set using a tolerance window of 0,2 min and 20 mDa. Data pretreatment was based on removingbackground noise and normalization by logarithmic transformation to reduce relatively large differences among the respective MFs abundances. MPP software enabled PCA and HCA of the data.

3. Result and Discussion

The determination of adulteration markers in pistachio nut was carried out in ESI-LC-QTOF system. First, green pea and pistachio nut extracts were injected to LC system. Figure 1. shows example total ion chromatograms of pistachio nut and green pea extracts.

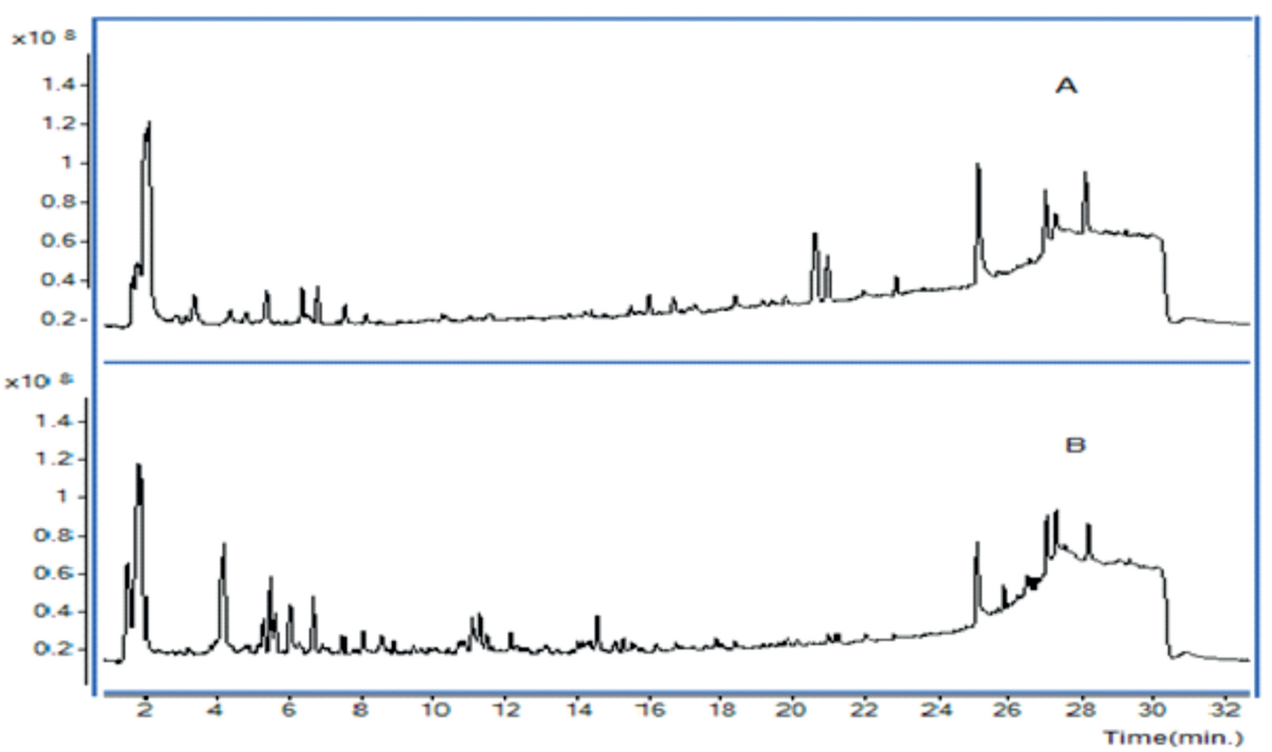

Figure 1. Total ion chromatograms obtained in negative ionisation mode (A) green pea and (B) pistachio nut

As can be seen in the total ion chromatograms, there are differences among the extracts. ESI (-)-MS fingerprints for the methanol:water extract of pistachio nut and green pea samples are visible in Figure 2.

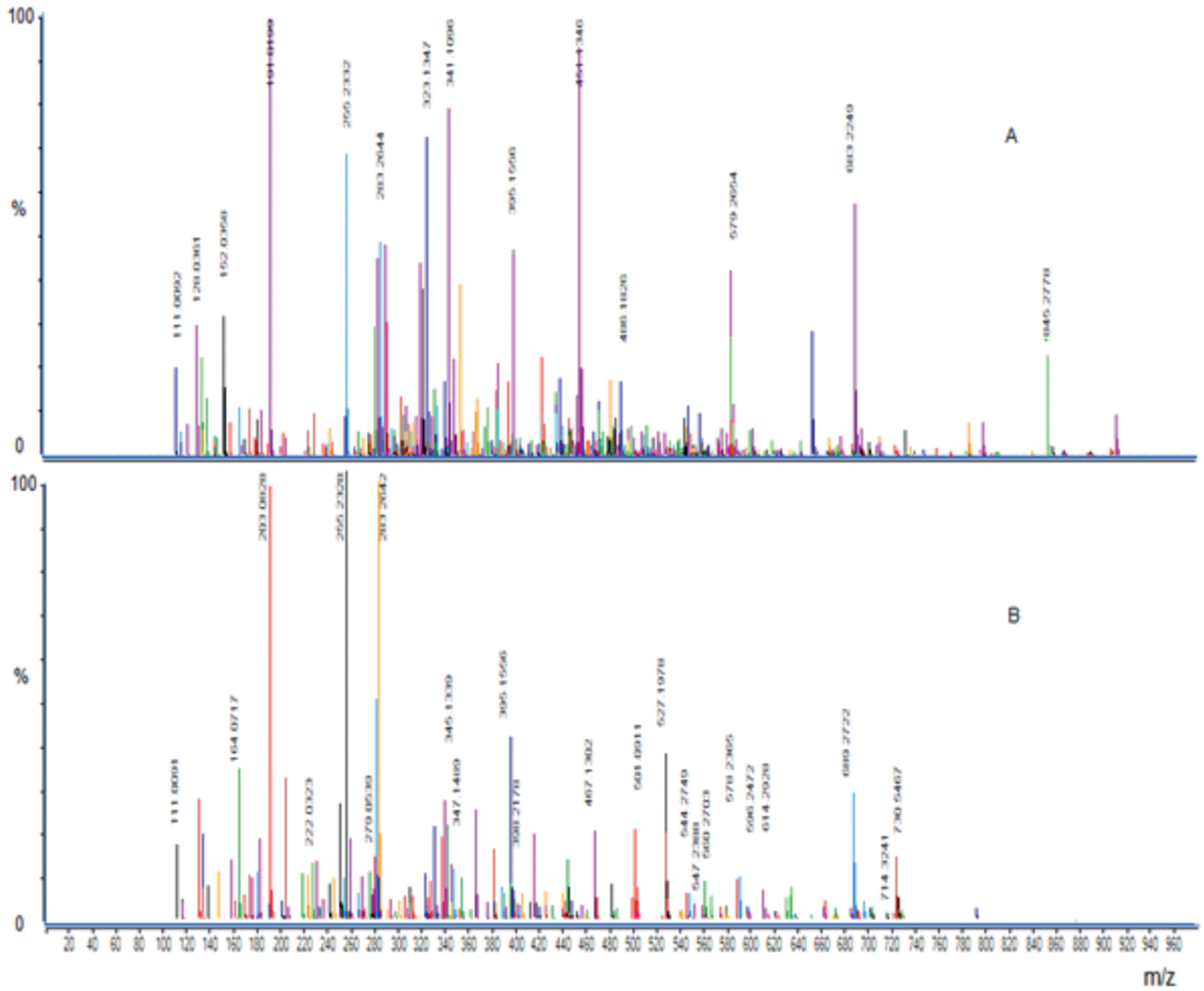

Figure 2. ESI(-)-MS fingerprints of (A) pistachio nut and (B) green pea

These mass spectra were very characteristic to each sample and the anions detected were most likely the deprotonated forms of polar component. Both pistachio nut and green pea extracts contain numerous polar components that form upon ESI a relatively complex and deprotonated molecules. These polar components consist of fatty acids, phenols, carboxylic acids and aliphatic alcohols (Tomaino et al. 2010; Tsantili et al. 2010). Some phenols were determined in both pistachio nut and green pea such as ferrulic acid, p-coumaric acid, quercetin, apigenin, naringin and hesperin.

For green pea major negative ion markers were determined using ESI(-)-MS fingerprints. The accurate mass information of precursor of these markers were used to calculate the proposed molecular formula of each marker. Atoms considered for the molecular formula calculation, were as follows: C (n≤50), H (n ≤100), O (n ≤20), N (n ≤20) and CI (n≤5). All suggested formulas have very good mass accuracy, less than 2 mDa (Table 1).

Table 1. The overview of characteristic marker compounds detected in green pea

Marker number	RT (min.)	(m/z)	Ion	Neutral mass	Probable ion elemental formula	Mass error(mDa)
1	19,07	222.0323	$[M-H]^-$	223.0402	$C_{11}H_{10}ClNO_2$	0.05
2	4,65	345.1339	$[M-H]^-$	346.1418	$C_{19}H_{22}O_6$	-0.51
3	6,26	347.1489	$[M-H]^-$	348.1548	$C_{19}H_{24}O_6$	0.79
4	4,18	501.0911	$[M-H]^-$	502.0990	$C_{20}H_{23}ClN_2O_{11}$	0.52
5	10,07	560.2703	$[M-H]^-$	561.2782	$C_{26}H_{43}NO_2$	0.67
6	8,34	547.2388	$[M-H]^-$	548.2467	$C_{25}H_{40}O_{13}$	0.60
7	11,71	502.2644	$[M-H]^-$	503.2723	$C_{24}H_{41}NO_{10}$	0.68
8	13,16	544.2749	$[M-H]^-$	545.2827	$C_{23}H_{35}N_{11}O_5$	-0.18
9	10,89	398.2178	$[M-H]^-$	399.2257	$C_{20}H_{33}NO_7$	0.52
10	7,28	279.0539	$[M-H]^-$	280.0618	$C_{13}H_{13}ClN_2O_3$	0.63
11	10,08	596.2472	$[M-H]^-$	597.2551	$C_{23}H_{36}ClN_{11}O_6$	-1.59
12	13,88	578.2365	$[M-H]^-$	579.2441	$C_{23}H_{34}ClN_{11}O_5$	-1.45
13	15,03	614.2928	$[M-H]^-$	615.3007	$C_{28}H_{45}N_3O_{12}$	0.74
14	18,31	714.3241	$[M-H]^-$	715.3320	$C_{33}H_{42}ClN_{15}O_2$	0.78
15	14,61	688.2722	$[M-H]^-$	689.2800	$C_{33}H_{43}N_3O_3$	-0.41

To evaluate the sensitivity of the technique, adulterated baklava samples containing different amounts of green pea and pistachio nut mixtures were prepared. After methanol extraction, adulterated baklava samples were searched specific negative ion of green pea. Table 2 lists the identified specific peaks between 1% and 10% (w:w) of the green pea specific negative ion found in different spiked preparations. In baklava samples adulterated with 5% and 10% green pea, all (15) specific peaks were detected. At 2,5 % level, only (8) of (15) negative ions were detected and at 1% level none of the specific peaks detected in the baklava samples. In the present technique green pea was detected at a minimum level of 2,5% in adulterated baklava samples.

Table 2. Specific green pea markers detection in a spiked baklava

Marker number	Green pea specific markers (m/z)	10% green pea /90% pistachio nut	5% green pea /95% pistachio nut	2.5% green pea /97,5% pistachio nut	1% green pea /99% pistachio nut
1	222.0323	Yes	Yes	Yes	No
2	345.1339	Yes	Yes	Yes	No
3	347.1489	Yes	Yes	Yes	No
4	501.0911	Yes	Yes	No	No
5	560.2703	Yes	Yes	Yes	No
6	547.2388	Yes	Yes	No	No
7	502.2644	Yes	Yes	Yes	No
8	544.2749	Yes	Yes	Yes	No
9	398.2178	Yes	Yes	No	No
10	279.0539	Yes	Yes	No	No
11	596.2472	Yes	Yes	No	No
12	578.2365	Yes	Yes	No	No
13	614.2928	Yes	Yes	No	No
14	714.3241	Yes	Yes	Yes	No
15	688.2722	Yes	Yes	Yes	No

Principal component analysis (PCA) and hierarchical cluster analysis (HCA) were performed with Mass Profiler Professional (MPP) software. PCA of the fingerprints (Figure 3) clearly separates pistachio nut and green pea in two well-defined groups. The discrimination between samples was performed using the whole ESI(-)-MS spectrum m/z range (100-1500). Three principal components were used to display the clustring of the samples. PC1, PC2 and PC3 were found to account for 70,02%, 6,05% and 4,41% of the total variance, respectively. Therefore, the total variance explained by PCs is (80,48)% at a confidence level of 95%. As a result, the PCA score plot shows that there is a large distance between pistachio nuts and green peas samples which means an indication of a successful separation.

Hierarchical cluster analysis (HCA) is a data analysis where natural grouping of objects is obtained. Grouping in HCA is based on similarities of samples (Chylinska et al. 2016). In this study, Ward's method, which is a very efficient method for the analysis of variance between clusters was applied using squared Eulidean distance. When HCA was applied to the ESI(-)-MS data, two groups emerged. The HCA is illustrated as a dendrogram in Figure 4 in which two main clusters are visible.

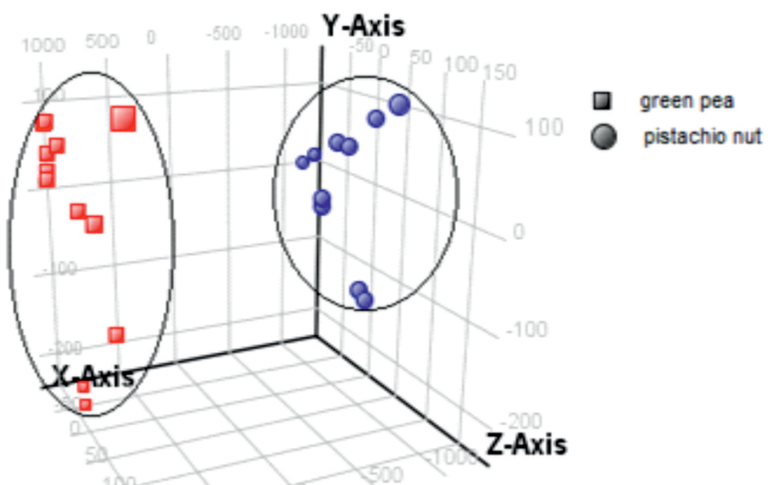

Figure 3. PCA score plot of pistachio nut and green pea samples obtained from the ESI(-)-MS

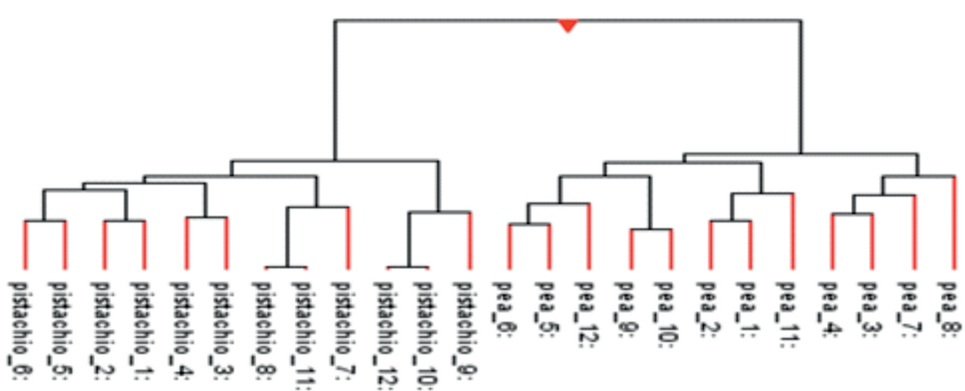

Figure 4. Dendrogram for pistachio nut and green pea samples obtained from the ESI(-)-MS

4. Conclusion

In conclusion, this study presents an analytical methodology by liquid chromatography-(quadrupole-time of flight)-mass spectrometry together with precise statistical analysis for detection of pistachio nut adulteration with green pea. By combining the advantages of ESI technique to LC-QTOFMS, the system becomes a useful and powerful tool to provide a characteristic profile of polar constituents. The ESI(-)-MS fingerprints were used to determine marker metabolites for green pea and green pea was detected at a minimum level of 2,5% in model food matrix "baklava". The specificityand sensitivity of this method can be used to confirmunambiguously the presence of green pea inadulterated pistachio nut powder and related products.

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